Neuroendocrine regulation of the fetal adrenal gland

David Charles Howe
MBChB (Edinburgh), BSc (Hons)

MRC Reproductive Biology Unit
Centre for Reproductive Biology
37 Chalmers Street,
Edinburgh EH3 9EW

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Contents

Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

David C Howe
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Abstract.

The physiological mechanisms initiating labour at term are poorly understood. In the fetal sheep activation of the fetal hypothalamo-pituitary-adrenal (HPA) axis in late gestation is essential for the birth of a viable fetus. Little is known of the ontogeny of the hypothalamic pathways controlling adrenocorticotrophin (ACTH) secretion in the fetus, nor of the metabolic or placental signals to which they are responding to initiate the surge in pituitary adrenal activity in late gestation. Exogenous agonists at central N-methyl-D-aspartate (NMDA) receptors stimulate ACTH secretion in the ovine fetus and the ACTH response to NMDA increases with gestation. These studies investigate the regulation of the HPA axis by endogenous amino acid transmitters acting through the NMDA receptor in the late gestation fetal sheep. The HPA axis responsiveness to neuropeptide Y (NPY), a neurotransmitter implicated in metabolic feedback to the HPA axis, is also investigated.

In the chronically cannulated late gestation sheep fetus CGP 37849, a competitive NMDA receptor antagonist, significantly decreased the ACTH and cortisol response to insulin hypoglycaemia stress on day 130 (term=145 days), and also reduced basal plasma concentrations on day 138, confirming that endogenous excitatory amino acid neurotransmitters acting through the NMDA receptor regulate ACTH secretion in the late gestation fetus. The coupling of the NMDA receptor to CRH and AVP secretion at the median eminence was investigated by a microdialysis approach. Pre-treatment with CGP 37849 attenuated the ACTH and cortisol responses to insulin hypoglycaemia, but not AVP secretion at the median eminence. It was not possible to detect CRH in the dialysate from any of the fetuses, but the failure of CGP 37849 to suppress AVP release suggests that NMDA receptors regulate ACTH secretion through CRH neurons. The potential for placental steroids to regulate the HPA axis response to NMDA was also investigated. Infusion of estradiol from day 120 for 96 h did not alter basal plasma ACTH or cortisol concentrations, pituitary sensitivity to AVP and

CRH, or ACTH or cortisol responses to NMDA, despite there being a significant suppression of plasma follicle stimulating hormone concentrations and an increase in plasma prolactin concentrations.

The role of NPY pathways to stimulate the HPA axis was also examined. In fetal sheep at day 125 gestation administration of 30 μg NPY into the lateral cerebral ventricle stimulated a small but significant increase in plasma concentrations of ACTH.

Overall, these studies suggest an important role for the NMDA receptor acting through CRH neurons in the late gestation increase in fetal HPA axis activity, however, the increase in HPA axis activity and sensitivity to NMDA are not dependent upon estrogen. Activation of the HPA axis may be due to metabolic signals since in late gestation NPY pathways can stimulate the HPA axis. A model is proposed where increasing sensitivity to NMDA reflects maturation of the hypothalamic pathways regulating ACTH secretion, and once these pathways are sufficiently developed metabolic signals are able to initiate parturition.

Abbreviations

ACTH	Adrenocorticotrophin
ANOVA	Analysis of the variance
AP-1	Activator protein-1
AVP	Arginine vasopressin
C	Centigrade
CBG	Corticosteroid binding globulin
cpm	Counts per minute
CRH	Corticotrophin releasing hormone
csf	Cerebrospinal fluid
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
emg	Electromyogram
FSH	Follicle stimulating hormone
GH	Growth hormone
GHRH	Growth hormone releasing hormone
h	Hour(s)
HPA	Hypothalamo-pituitary-adrenal axis
11βHSD	11β-hydroxysteroid dehydrogenase
icv	Intracerebroventricular(ly)
IGF-1	Insulin-like growth factor-1
IRMA	Immunoradiometric assay
IU	International units
iv	Intravenous(ly)
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
M	Mole
mCi	Millicurie
min	Minute(s)
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NIL	Neurointermediate lobe
NMDA	N-Methyl-D-Aspartate
NPY	Neuropeptide-Y
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PRL	Prolactin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
rpm	Revolutions per minute
SF-1	Steroidogenic factor-1
SON	Supraoptic nucleus
v/v	Volume for volume
w/v	Weight for volume

1

Maturation and function of the fetal hypothalamic pituitary adrenal axis

1.1 Introduction

"Deform'd, unfinish'd, sent before my time
Into this breathing world, scarce half-made up,
And that so lamely and unfashionable
That dogs bark at me, as I halt by them;"
Richard III (Shakespeare)

The transition at birth to extra-uterine life places remarkable demands upon the developing fetus. In an instant it must be able to breathe for itself, and then in the longer term take in nourishment and maintain internal homeostasis. The timing of birth at a point where fetal organs have matured sufficiently to be able to support independent life is critically important. Nearly 60% of human infants born at 26 weeks will not survive their first few months, and in those that do, the incidence of clinically apparent neurological deficit is around 25% (Morrison and Rennie 1997). Survival and morbidity improve steadily with increasing gestational age but even infants born at 35 weeks are at risk of transient tachypnoea of the newborn and respiratory distress syndrome due to lung immaturity (Morrison, Rennie et al. 1995). It is estimated that about 0.5% of births occur before 28 weeks, and 5-10% before 37 completed weeks (Morrison and Rennie 1997). Conversely pregnancy carried beyond term is associated with increased risk of stillbirth as the spectres of oligohydramnios and diminishing placental reserve supervene

(Grant 1994; Hilder, Costeloe et al. 1998). An understanding of why birth occurs early in some pregnancies and late in others and how this is linked with fetal maturation is of major clinical importance for the development of effective strategies for the prevention of preterm birth, the promotion of fetal maturity and the induction of labour.

It has been known for several decades that the fetal adrenal is intimately linked with adaptation to extrauterine life: increased secretion of corticosteroids by the fetal adrenal in late gestation promotes lung maturity in preterm animals and human infants as well as maturing many other organ systems (Liggins 1976; Fowden, Li et al. 1998). In species such as the sheep it is clear also that products of the fetal adrenal initiate the endocrine cascade that leads to labour, so ensuring the birth of an appropriately mature fetus.

Inevitably there are species differences in some of endocrine events of parturition but there are also considerable similarities reflecting the common evolution of the utero-cervical canal from a primitive genital pore (Ryan 1977). Increasing fetal corticosteroid production turns out to be a conserved signal used by all mammalian species to orchestrate the maturational changes needed for extra uterine survival with birth (Liggins 1983; Fowden, Li et al. 1998), just as a change in the balance of progesterone to estrogen in the maternal circulation regulates uterine activity and cervical compliance (Lye 1994).

Despite some three decades of research the processes which lead to increasing secretion of steroids from the fetal adrenal cortex to initiate parturition remain enigmatic. This thesis examines aspects of hypothalamic regulation of the fetal adrenal cortex in late gestation in the sheep. The following review chapter considers how the fetal sheep hypothalamo-pituitary-adrenal axis initiates parturition and draws parallels with the primate fetus, then describes the basic organisation of the hypothalamo-pituitary-adrenal axis and the functional development of the axis in fetal life, the putative peripheral signals which may drive increasing adrenal steroid secretion through hypothalamic pathways, and lastly what little is known of the hypothalamic pathways themselves. From this a series of hypotheses are advanced about the role of excitatory amino acid neurotransmitters and

Neuropeptide Y pathways in the regulation of the fetal adrenal The remainder of the thesis details the experimental work involved in testing these hypotheses.

1.2 The fetal hypothalamo-pituitary-adrenal axis and parturition

1.2.1 The ovine model

Perhaps the best insight into the mechanisms controlling fetal maturation and birth come from the sheep. Serendipitous observation revealed a major role of fetal adrenal steroids in initiating labour when it was noted that sheep grazing on pokeweed (*Veratrum californicum*) early in pregnancy gave birth to deformed fetuses which were carried well beyond term (Binns, James et al. 1964). Investigation showed that toxins from the pokeweed disrupt normal development of the fetal brain and pituitary and result in hypoadrenalism. That fetal adrenal cortisol is crucial for birth has been shown by subsequent experiments demonstrating that lesions of the hypothalamus, pituitary or adrenal all prevent labour (Liggins, Kennedy et al. 1967; Drost and Holm 1968; Gluckman, Mallard et al. 1991; McDonald and Nathanielsz 1991). There is good evidence for increasing activity of the fetal hypothalamus, and increasing circulating concentrations of adrenocorticotrophin (ACTH) and cortisol in the days before birth (Norman, Lye et al. 1985; Hoffman, McDonald et al. 1991). Furthermore exogenous cortisol or ACTH given directly to the fetal sheep will induce premature labour (Liggins, Kennedy et al. 1967; Liggins 1968). It was also noted that those lambs delivered prematurely survived because of accelerated production of lung surfactant. Indeed, the fetal adrenal through the secretion of cortisol is a major switch for many organ systems preparing them for extra uterine life (Liggins 1976). By acting both as a trigger for labour and as a signal inducing neonatal patterns of tissue gene expression, the ovine fetal adrenal ensures the birth of an appropriately mature lamb.

The action of cortisol to promote labour in the sheep is bought about by a change in the placental production of the steroid hormones estradiol and

progesterone (Liggins, Grieves et al. 1972). Early in gestation the placenta becomes established as the major source of progesterone in maternal and fetal circulations (Liggins 1983). Throughout pregnancy uterine activity is suppressed by progesterone (Li, Perezgrovas et al. 1991; Gazal, Li et al. 1993). Yet at term all this changes: cortisol induces placental C17-20 lyase and 17-α hydroxylase activity which allows the placenta to metabolise progesterone through to estradiol (Mason, France et al. 1989; Rose and JP 1994). There is also a modest cortisol dependent increase in aromatase activity (Mason, France et al. 1989). The placental output of progesterone falls over several days as estradiol production rises. Concentrations of progesterone in the maternal circulation decline from about 12 ng/mL a week prior to delivery to 1ng/mL on the day of delivery (Bassett, Oxborrow et al. 1969). At the same time the concentration of estradiol increases markedly (Challis 1971). This change in the steroid environment from progesterone to estrogen dominance leads to the onset of uterine muscular contraction and remodelling of cervical connective tissue. Besides inducing the placental enzymes for estrogen synthesis, the ovine fetal adrenal also contributes to the surge in estrogen through the production of androgenic precursors that the placenta aromatises to estrogen. In fact at term as much as one third of estrogen production is from substrates provided by the fetal adrenal gland (Mitchell, Lye et al. 1986).

The precise way in which the steroid hormones impinge upon the complex regulatory networks controlling myometrial activity are not fully understood. Antagonism or removal of progesterone during pregnancy results in abortion or labour (Gazal, Li et al. 1993). Clearly in these circumstances there is no rise in estradiol yet the utero-cervical canal undergoes many if not all of the changes seen in spontaneous labour. Conversely, estradiol infused in high doses will induce labour even though progesterone production dose not fall (Cahill, Knee et al. 1976). At a molecular level many of the structural proteins, receptors and hormones controlling the myometrium are influenced in opposite ways by estrogen and progesterone (Lye 1994).

1.2.2 Primates (and other species)

An increase in the concentration of corticosteroid in the fetal circulation late in gestation has been demonstrated in the numerous species including the goat (Currie and Thorburn 1977), cow (Hunter, Fairclough et al. 1977)) horse (Silver 1990), rat (Martin, Cake et al. 1977; Chatelain, Dupouy et al. 1980; Boudouresque, Guillaume et al. 1988), rabbit (Hummelink and Ballard 1986), pig (Dvorak 1972), baboon (Pepe and Albrecht 1984), and rhesus monkey (Jaffe, Seron-Ferre et al. 1978). Direct evidence for a rise in human fetal corticosteroids with advancing gestation has been provided by studying the levels of corticosterone sulphate in the maternal circulation in a woman who had undergone bilateral adrenalectomy for Cushing's syndrome (deFencl, Stillman et al. 1980). This is supported by measurements of circulating corticosteroids in blood obtained at cordocentesis (Lopez-Bernal and MacKenzie 1987; Nahoul, Daffos et al. 1988; Nahoul, Daffos et al. 1989; Donaldson, Nicolini et al. 1991).

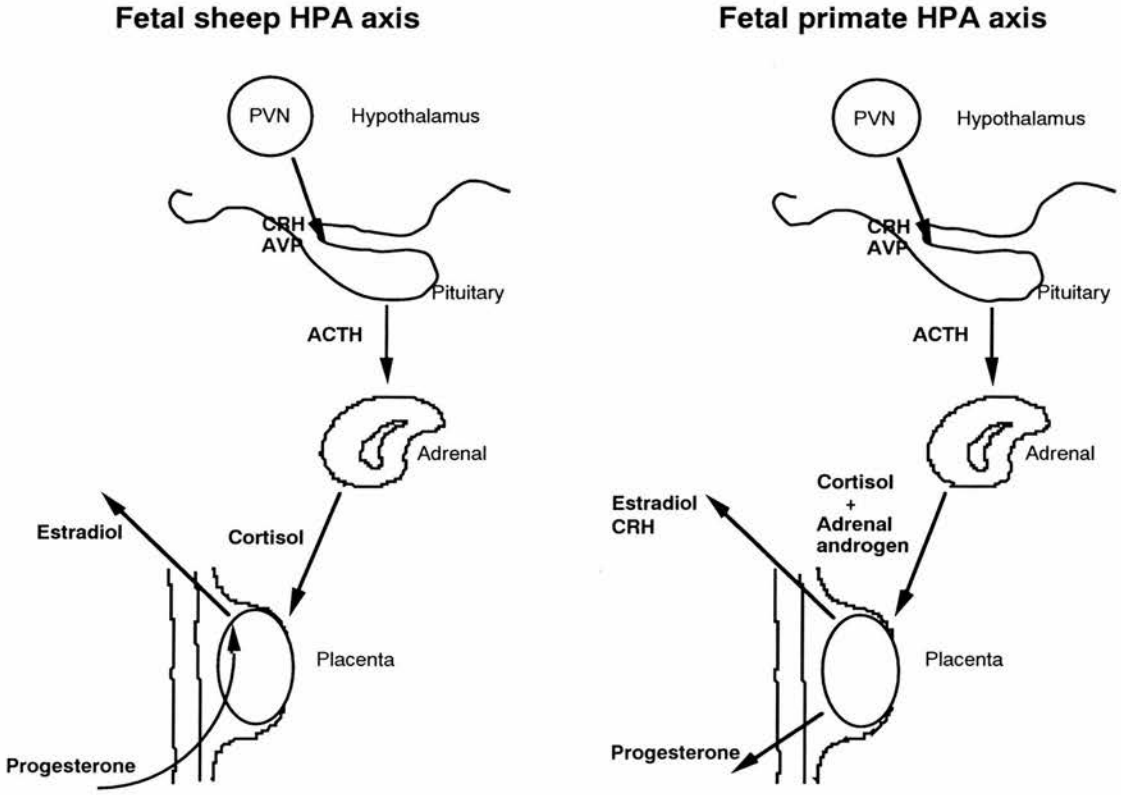
In humans and other primates, unlike the sheep, the placental enzymes needed for estrogen synthesis are constitutively expressed and are not cortisol inducible (Liggins 1983; Albrecht and Pepe 1990). The fetal adrenal still contributes to the increase in circulating estrogens by providing the precursors for placental estrogen synthesis so that in primates, as in other mammals, the balance of estrogen and progesterone changes in late gestation. In humans it is estimated that near term 50% of estradiol and 90% of estriol in maternal plasma is derived from precursors provided by the fetal adrenal (Sitteri and MacDonald 1966; Barlow, Goldstein et al. 1967). Infusion of androgenic precursors in late pregnancy in the rhesus macaque will advance the onset of labour demonstrating the importance of the fetal adrenal for primate parturition (Mecenas, Giussani et al. 1996).

Cortisol may have some facilitatory role in the onset of labour in primates (Challis and Brooks 1989; Guller, Wozniak et al. 1993; Challis, Matthews et al. 1995; Karalis, Goodwin et al. 1996). The major function of cortisol in the human fetus, however, is probably to prepare the lungs and other organs for birth (Liggins 1976; Crowley, Chalmers et al. 1990). It is

perhaps worth noting that even at 37 weeks of gestation the incidence of respiratory distress in the newborn is higher than infants delivered at full term (Morrison, Rennie et al. 1995).

In other species (for example, the goat, cow, pig, rat and rabbit) the principal site of progesterone synthesis is the corpus luteum (Poyser 1995). Parturition is initiated by luteolysis which results in a fall in progesterone concentrations in the maternal circulation. In the goat, cow and pig the fetus signals parturition with an increase in cortisol which stimulates placental estrogen synthesis. In turn, an increase in estrogen stimulates the release of the luteolytic hormone prostaglandin F2α (PGF2α) from the placenta and decidua (Poyser 1995).

Diagram summarising the contributions of the fetal HPA axis to the endocrine events at parturition in the sheep and primate.



1.3 Basic organisation of the hypothalamo-pituitary adrenal axis

1.3.1 The adrenal gland

Steroid synthesising cells in the adrenal cortex are essential for survival for if the adrenal is removed or destroyed by disease processes, intermediary metabolism, fluid balance, cardiovascular and immune functions gradually deteriorate and death supervenes (Orth, Kovacs et al. 1992). The principal steroid products of the adrenal are corticosteroids, mineralocorticoids and sex steroid hormones. Within the adrenal cortex there is a spatial distribution of the different cells expressing the enzymes necessary for synthesis of each of the principal steroid hormones (Orth, Kovacs et al. 1992). Mineralocorticoid synthesising cells are clustered together in groups in the zona glomerulosa near the outer capsule of the gland. Deeper in cells are arranged in rather irregular strings referred to as the zona fasiculata and secrete corticosteroids. The inner layer of the adrenal cortex, termed the zona reticularis, abutting on the adrenal medulla, consists of sex steroid secreting cells.

The control of adrenal corticosteroid production involves cells located in the pituitary and hypothalamus, along with the central nervous system pathways afferent to the hypothalamus; Together these are referred to as the hypothalamo-pituitary adrenal (HPA) axis. Stimulatory drive to adrenal corticosteroid and androgen secretion is provided by the peptide hormone ACTH secreted by corticotroph cells of the anterior pituitary. Specific adenylate cyclase coupled receptors in the adrenal bind ACTH (Stocco and Clark 1996). Adrenocorticotrophin not only stimulates steriodogenesis by mobilising intracellular cholesterol, but increases expression of steroidogenic enzymes and mitochondrial steroid acute regulatory protein (Clark and Stocco 1996; Stocco and Clark 1996).

1.3.2 The pituitary gland

The pituitary gland can actually be divided into three separate regions (Orth, Kovacs et al. 1992). The posterior pituitary consists of the neurosecretory axons arising from magnocellular oxytocin and vasopressin containing neurons in the supra-optic and paraventricular nuclei. The anterior pituitary consists of glandular cells served by the hypothalamo-hypophysial portal tract. There is little innervation of the anterior pituitary so that secretion is regulated principally through endocrine and paracrine signals. The third region of the pituitary is the neurointermediate lobe (NIL), a structure wrapped around the bundle of neurosecretory nerve fibres passing to the posterior pituitary. Cells of the NIL are thought to be directly innervated by dopaminergic neurons with cell bodies located in the tuberal-hypophysial region (Lookingland, Farah et al. 1985).

Anterior pituitary corticotrophs synthesise and secrete ACTH. The 39 amino acid sequence of the peptide is highly conserved across species, though full biological activity seems to reside with amino acids 1-24 of the sequence (kapas, Cammas et al. 1996). The gene for ACTH actually encodes the sequence of a larger precursor molecule, pro-opiomelanocortin (POMC), from which ACTH is derived by proteolytic cleavage (Eipper and Mains 1980). The POMC gene is also expressed in melanotrophs of the NIL of the pituitary, but is post translationally processed to alternative products.

1.3.3 The hypothalamus

The secretion of ACTH from the anterior pituitary is stimulated by the hypothalamic releasing factors arginine vasopressin (AVP) and corticotrophin releasing hormone (CRH) (Plotsky 1991; Antoni 1993; Whitnall 1993). These two peptides are secreted into the specialised hypothalamic-hypophysial portal system at the median eminence by neurosecretory neurons located in the parvocellular portion of the hypothalamic paraventricular nucleus (PVN). Magnocellular neurons in the PVN and supraoptic nucleus (SON) also contain AVP, and in the rat these neurons secrete AVP in the median eminence “en

passant" from varicosities on the axons as they pass to the posterior pituitary (Antoni 1993). It is not known if similar pathways are present in other mammals.

The fine control of ACTH secretion is even more complex, though, since a number of other peptides found in portal plasma can stimulate or inhibit ACTH secretion (Plotsky 1991; Antoni 1993). It has also been demonstrated in culture that basal electrical activity and secretion by some pituitary corticotroph can continue in the absence of hypothalamic releasing factors, though AVP and CRH do alter the pattern of electrical activity and presumably enhance ACTH secretion from these cells (Kwiecieu and Hammond 1998).

Many brain structures are involved in integrating the internal and external stimuli to appropriately control corticosteroid secretion. Responses to metabolic stress and to injury are probably mediated through brainstem and circumventricular structures and the hypothalamic PVN directly, wheras responses requiring interpretation in the light of previous experience involve pathways in the prefrontal cortex, hippocampus and amygdala (Antoni 1993; Whitnall 1993; Herman, Prewitt et al. 1996).

1.3.4 Corticosteroid action and negative feedback

Feed back by adrenal corticosteriods at the level of the central nervous system and pituitary completes a simple feedback loop limiting further drive to the adrenal gland. Acutely, corticosteroids decrease ACTH secretion, and over the course of several hours decrease CRH, AVP and POMC expression (Drouin, Sun et al. 1989; Reichardt and Schutz 1998). Rapid actions of steroids in excitable tissues probably occur through modulation of cell surface receptors (Revelli, Massobrio et al. 1998). The slower effects of corticosteroids on the genome are classically mediated through intracellular receptors which bind to the genome and regulate gene expression (Evans 1988; Beato 1989; Tsai and O'Malley 1994; Beato, Herrlich et al. 1995).

The intracellular events regulating corticosteroid action on the genome are complex and only partly characterised. Separate corticosteroid

(glucocorticoid) and mineralocorticoid receptors exist, but in fact corticosteroids are capable of binding to both receptors *in vitro* with near equal affinity (Funder 1997). In classical mineralocorticoid sensitive tissues, such as the renal distal tubule, corticosteroids are prevented from binding mineralocorticoid receptors *in vivo*, by an intracellular enzyme, 11-β hydroxysteroid dehydrogenase (11βHSD), which serves to protect the mineralocorticoid receptor by metabolising corticosteroids (Funder 1997). During studies on the 11βHSD enzyme it has become apparent that there are at least two different isoforms (Gomez-Sanchez and Gomez-Sanchez 1997), and that in non-mineralocorticoid receptor expressing tissues, and in the brain, an isoform with lower affinity, and which is bidirectional operates to regulate intracellular corticosteroid concentrations. Since this latter isoenzyme is bidirectional it may be important for maintaining corticosteroid concentrations rather than simply inactivating them.

Glucocorticoid and mineralocorticoid receptors are transcription factors, which once they have bound biologically active steroid form a dimer with another transcription factors before binding to the promoter sequence of corticosteroid regulated genes. In the case of the glucocorticoid receptor it appears that homodimers with other glucocorticoid receptors, or homodimers with other steroid receptors, or other transcription factors, may be formed, and that the different dimers have different effects on gene expression (Reichardt and Schutz 1998). An additional complexity is that full activity of the transcriptional complex is determined by coactivators and corepressors (Chen and Evans 1995; Onate, Tsai et al. 1995; Horwitz, Jackson et al. 1996).

There is debate about the relative importance of mineralocorticoid and glucocorticoid receptors in the various brain regions implicated in corticosteroid feedback (de Kloet, Vrengdenhil et al. 1998). It has been suggested that severe physiological stress activates the HPA axis through brainstem and circumventricular structures, wheras stressors requiring interpretation in the light of previous experience involve pathways in the prefrontal cortex, hippocampus and amygdala (Herman, Prewitt et al. 1996) and all these regions express glucocorticoid and mineralocorticoid receptors

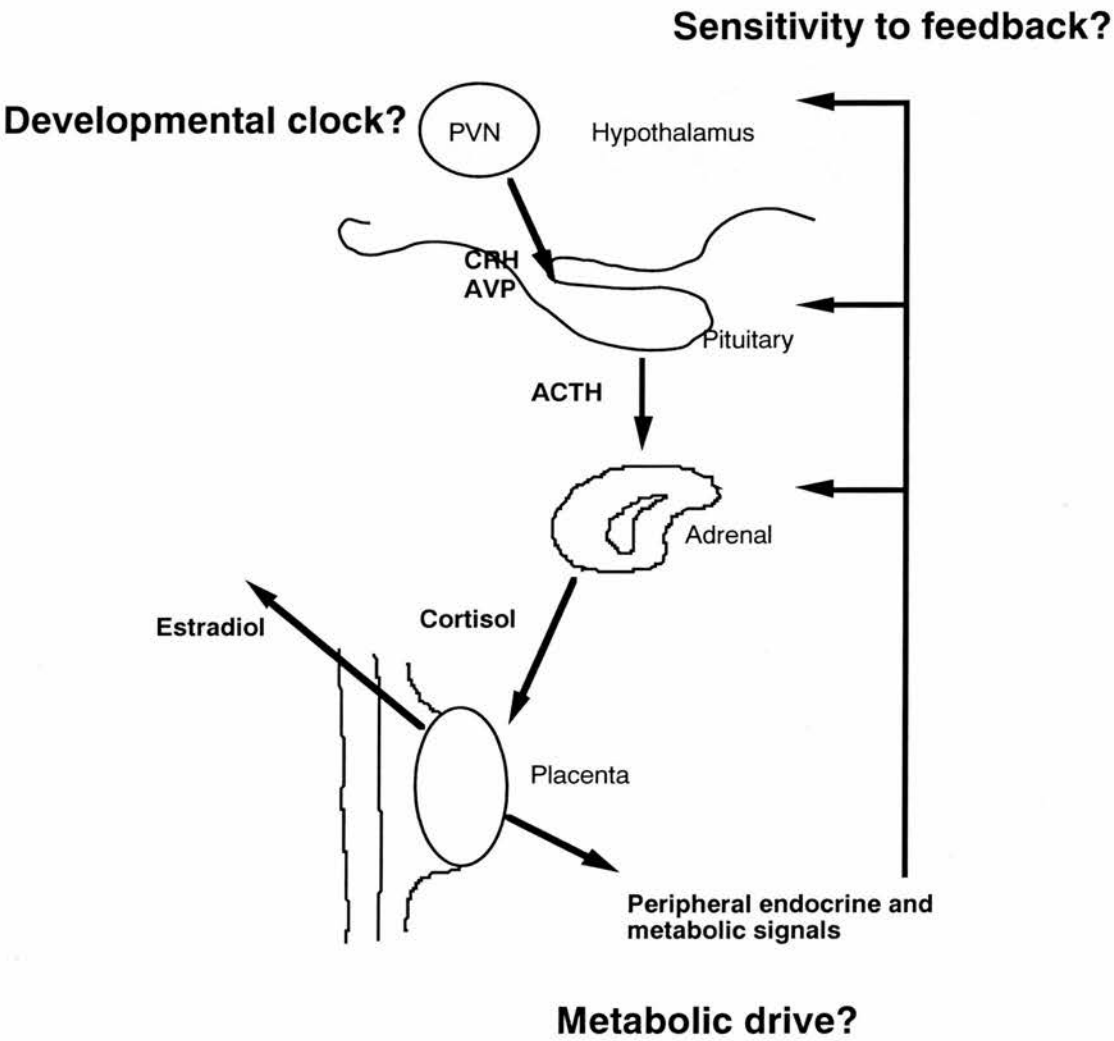
(de Kloet, Vrengdenhil et al. 1998). To maximise adaptability in the system it is likely that the actions of glucocorticoids will be different in the different brain regions.

1.3.5 The fetal HPA axis: unresolved issues

The concomitant rise in plasma ACTH and cortisol seen in late gestation is a paradox, since normally elevated corticosteroid concentrations suppress further ACTH synthesis and secretion. Two simplistic explanations may be evoked: firstly that there is a reduction in negative feedback sensitivity occurring specifically around the time of parturition, and secondly that there is increasing hypothalamic drive to ACTH secretion which overcomes cortisol negative feedback. Increasing drive is not simply a resetting of the set-point around which the level of cortisol is regulated, because it implies that once the stimulus for increased ACTH secretion is removed, the system will return to its original state. A rather more complicated explanation is that the increase in ACTH derives from a non-pituitary source that cannot be suppressed by corticosteroids. In the discussion that follows the functional development of the hypothalamus, pituitary and adrenal are described and the evidence for altered negative feedback sensitivity considered.

Aside from trying to understand the mechanisms allowing a sustained increase in corticosteroid secretion, the issue of what initiates a decline in feedback sensitivity, or increasing hypothalamic drive still remains unresolved. The characteristic species specific duration of gestation suggests some counting or timing process is involved, and certainly genetically programmed patterns of development could be interpreted as an intrinsic biological clock. An alternative explanation is that signals from the mother or placenta, perhaps relating to fetal size or maturity, provide the stimulus for ACTH and cortisol secretion. In other words, peripheral epigenetic factors regulate the final timing of parturition. The last sections of this review consider metabolic and placental signals which may drive the hypothalamus, and finally the hypothalamic pathways themselves that may be important for the regulation of ACTH secretion in the fetus.

The diagram below summarises the putative mechanisms leading to increasing fetal adrenal steroid secretion and the initiation of parturition.



1.4 Development of the hypothalamus

1.4.1 Differentiation of the paraventricular nucleus

The co-ordinated response of the HPA axis during fetal and adult life depends upon the establishment of properly connected neuroendocrine pathways during development. Developing PVN neurons must establish synaptic contact with incoming axons from both intrinsic hypothalamic neurons and remote brain regions, and at the same time form neurosecretory contacts with developing median eminence capillaries. Similarly, the specialised hypothalamo-hypophyseal portal vasculature must form correctly to allow the hypothalamus to exert control over anterior pituitary corticotrophs. Much of the embrogenesis of the HPA axis is complete relatively early in gestation but it is not clear when the forming hypothalamic-hypophyseal unit establishes functional control over adrenal secretion. By about 0.7 of gestation, however, circulating fetal adrenal steroids start to increase and this does not happen where the hypothalamic neurons are absent, for example in anencephalics or following experimental destruction, indicating that some functional hypothalamic control over the adrenal has been established by this period of gestation (Allen, Greer et al. 1974; McDonald, Rose et al. 1988). Equally, full maturation of the HPA axis and its integration with the autonomic and behavioural responses to stress may not be complete until many months into post-natal life (Meaney, Tannenbaum et al. 1994).

The genes involved in the differentiation of PVN neurosecretory neurons and the migration of their axons to the median eminence have not been identified. Mapping of the formation of hypothalamic nuclei using tritiated thymidine incorporation into dividing neural progenitor cells reveals that in primates, rodents and the cat the hypothalamic nuclei are formed by 0.3, 0.7, and 0.5 of gestation respectively (Ifft 1972; Wyss and Sripandidkulchai 1985; Van Eerdenburg and Rakic 1994). Early in development most cells of the hypothalamus (and other forebrain regions) express the homeodomain regulatory protein POU 4 but later POU 4 expression is restricted to PVN neurons (Mathis, Simmons et al. 1992; Treacy

and Rosenfeld 1992). The protein is a transcription factor that by analogy to other homeodomain proteins plays a role in pattern formation in the embryo. The restriction to PVN neurons in later life suggests that it may also be important in directing the differentiation of these cells. Interestingly POU 4 may bind to the CRH promoter region and could thus play a role in switching on or maintaining CRH synthesis (Treacy and Rosenfeld 1992). The promoter regions of the gene for CRH also contains an estrogen response element consensus sequence (Vamvakopoulos and Chrousos 1994) so that the intriguing possibility exists that estrogen, provided by the placenta may have a role in the differentiation of these neurons.

1.4.2 Expression of AVP and CRH message and peptide

Gestation in sheep is about 145 days with some minor strain differences. It is not known when AVP and CRH first make their appearance in the hypothalamus of the fetal sheep but protein can be detected from at least 40 days gestation by immunocytochemistry. Immunoreactive fibres are seen in the median eminence at a similar time (Levidiotis, Oldfield et al. 1987; Watabe, Levidiotis et al. 1991). The hypothalamic content of extractable immunoreactive AVP and CRH increases from about day 60, when peptide is first detected, to around day 135 (Watabe, Levidiotis et al. 1991; Currie and Brooks 1992; Keiger, O'Steen et al. 1994). There is a small decrease in CRH and AVP content at term and then content increases again in the neonatal lamb (Brieu, Tonon et al. 1989). The total hypothalamic content of AVP is about 5 times that of CRH from early in gestation to about 125 days, but then the relative amounts of CRH increase through to term (Brieu, Tonon et al. 1989). Since not all of the AVP and CRH containing neurons of the hypothalamus project directly to the median eminence it is difficult to know how much of this peptide content relates to ACTH secretion.

When messenger ribonucleic acid (mRNA) content is examined by in situ hybridisation it is found that expression of CRH gene in the PVN parallels the changes seen in peptide content with the exception of a pool of laterally placed neurons which maintain high expression of CRH message upto

parturition. (Myers, Myers et al. 1993; Matthews and Challis 1995). In contrast, others using an RNAase protection assay find an increase in hypothalamic CRH message at term (Keiger, O'Steen et al. 1994). There is no change in the expression of AVP message in parvocellular neurons of the ovine fetal hypothalamus in animals in labour compared with non-labouring late gestation fetuses (Matthews and Challis 1995).

1.4.3 Hypothalamic regulation of the fetal pituitary

The critical role of the ovine fetal hypothalamus in ACTH secretion during development has been demonstrated in hypothalamo-pituitary-disconnected and PVN lesioned animals. In the fetal sheep, bilateral destruction of the PVN abolishes the preparturient surge of ACTH and cortisol, and prevents birth (McDonald, Rose et al. 1988; Gluckman, Mallard et al. 1991; McDonald and Nathanielsz 1991). Similarly disconnection of the hypothalamus from the pituitary prevents the preparturient rise in cortisol and postpones birth (Antolovich, Clarke et al. 1990; Antolovich, McMillen et al. 1991; Canny, Young et al. 1998). Replacement of ACTH in hypophysectomised sheep by constant low dose infusion of ACTH results in increasing cortisol secretion (Jacobs, Young et al. 1994; Poore, Young et al. 1998). Together these findings imply that the fetal hypothalamus is obligatory for the secretion of biologically active ACTH and hence the late gestation increase in cortisol, although the adrenal may not actually need an increase in circulating ACTH to mount an increase in cortisol.

In adult and fetal sheep hypothalamo-pituitary disconnection has been developed as a model to examine hypothalamic regulation of pituitary secretion. Following hypothalamo-pituitary disconnection in the ovine fetus there is a failure to initiate the pre-parturient surge in cortisol and consequently pregnancy is prolonged (Antolovich, McMillen et al. 1991; Deayton, Young et al. 1994; Phillips, Ross et al. 1996). The failure of the cortisol surge seems to be due to immaturity of the fetal adrenal steroidogenic enzyme systems (Phillips, Ross et al. 1996; Ross, Phillips et al. 1997), though low levels of cortisol secretion are maintained (Canny, Young et al. 1998).

Studies examining plasma ACTH concentrations after hypothalamo-pituitary disconnection have produced somewhat conflicting results. Measurement of ACTH concentration by radioimmunoassay (which recognise only one epitope on the ACTH molecule and so may cross react with smaller and larger molecular weight POMC products) generally finds that there is no change or a modest increase in ACTH immunoreactivity following disconnection (Ozolins, Young et al. 1990; Antolovich, McMillen et al. 1991; Antolovich, McMillen et al. 1992; Ozolins, Young et al. 1992; Canny, Young et al. 1998). Similar observations have been made in adult sheep and interpreted as evidence of an inhibitory hypothalamic regulation of basal ACTH secretion (Engler, Pham et al. 1988; Mercer, Clements et al. 1989). When a specific two-site immunoradiometric assay is used, basal plasma concentrations of ACTH do not differ between intact and hypothalamo-pituitary disconnected fetuses (Deayton, Young et al. 1994; Phillips, Ross et al. 1996; Ross, Phillips et al. 1997). Interestingly, ACTH secretion in the hypothalamo-pituitary disconnected fetus continues to be pulsatile and pulse characteristics are not significantly different from intact fetuses (Canny, Young et al. 1998).

The observations in the hypothalamo-pituitary disconnected fetuses are in contrast to the findings after PVN lesioning where the late gestation increase in ACTH is abolished once PVN neurosecretory neurons are destroyed (McDonald, Rose et al. 1988; McDonald and Nathanielsz 1991). It is not clear why basal secretion fails after PVN lesioning, but continues after hypothalamo-pituitary disconnection. One explanation is that after transection of the pituitary stalk, and in the absence of dopaminergic stimulation, the melanotrophs of the NIL synthesise ACTH. Evidence from transgenic mice supports this interpretation: in the dopamine (D2) receptor knockout mouse, the expression of mRNA for POMC in the NIL is increased, and there is also a change in the expression of the convertase enzymes processing POMC, so that the NIL secretes more ACTH (Saiardi and Borreli 1998).

Supra-pituitary pathways do regulate stimulated ACTH secretion. Stress responses seem to develop relatively early in utero in the ovine fetus and require an intact hypothalamic-pituitary connection. Between 74 and 84

days the ovine fetus is capable of a modest ACTH and cortisol response to acute haemorrhage (McFarlane, Potocnik et al. 1995). The stress responses to hypoglycaemia, nitroprusside-hypotension and prostaglandin E2 are prevented by hypothalamo-pituitary disconnection (Ozolins, Young et al. 1992; Young, Loose et al. 1996). Glucocorticoids will suppress plasma ACTH concentrations in intact but not in hypothalamo-pituitary disconnected fetuses at day 138 gestation (Ozolins, Young et al. 1990).

The principal hypothalamic peptides responsible for stimulating the release of ACTH from the anterior pituitary corticotroph are AVP and CRH. Immunoneutralisation studies in adult sheep suggest that AVP and CRH are involved in different modes of ACTH secretion, so that CRH is important for maintaining ACTH secretion by the pituitary gland in the unstressed state, wheras AVP is more important for the stress response (Guillaume, Conte-Devolx et al. 1992; Guillaume, Conte-Devolx et al. 1992). In the ovine fetus in late gestation, antagonism of AVP does not affect resting levels of ACTH but does impair the response to stress (Apostolakis, Long et al. 1991). More recently, it has been demonstrated that infusion of a specific CRH receptor antagonist into the ovine fetus in late gestation prevents the normal rise in plasma ACTH concentrations (Chan, Falconer et al. 1998). Further evidence that CRH neurons are important in driving the pituitary adrenal axis comes from the CRH-knockout transgenic mouse, where homozygous fetuses are born with evidence of failed lung maturation which can be reversed with glucocorticoid treatment (Muglia, Jacobson et al. 1995). These observations imply that the hypothalamic drive to the fetal pituitary in late gestation is mediated through CRH containing neurons in the parvocellular PVN.

1.5 Development of the pituitary

1.5.1 Differentiation and maturation of the corticotroph

The pituitary corticotroph differentiates and matures in parallel with the developing hypothalamus (Trier and Rosenfeld 1996). In mice, a common progenitor gives rise to the five specialised cell types secreting the

polypeptide hormones of the anterior pituitary but there is a temporal and spatial pattern to the appearance of each of these (Simmons, Voss et al. 1990; Japon, Rubinstein et al. 1994). The earliest cells to appear are the corticotrophs and these seem to be distributed mostly around the periphery of the gland (Japon, Rubinstein et al. 1994). Proopiomelanocortin message and immunoreactivity are readily detectable at 60 days gestation in the anterior pituitary of the ovine fetal pituitary and, as in the mouse, are situated peripherally (Matthews, Han et al. 1994). During development the volume of the pituitary increases and at the same time the number of ACTH immunopositive cells increases from about 6% at day 60 to 10% by day 100, whereafter the number of ACTH immunopositive cells remains relatively constant until delivery (Matthews, Han et al. 1994). The morphology of ACTH immunoreactive cells changes during gestation. From at least day 90 onwards, the ovine fetal pituitary contains both large columnar cells staining weakly for ACTH that have been termed "fetal corticotrophs" and smaller stellate cells staining intensely for ACTH termed "adult corticotrophs" (Perry, Mulvoque et al. 1985). In later gestation the adult type corticotroph becomes predominant. Adrenal peptide or steroid hormones may be important in initiating differentiation to the adult type corticotroph since fetal adrenalectomy prevents the switch from fetal to adult corticotroph (Antolovich, Perry et al. 1989). Hypothalamo-pituitary disconnection also disrupts development of the corticotroph, though this may be a consequence of the failure of adrenal maturation (Antolovich, McMillen et al. 1991).

The genes controlling the differentiation and maturation of the corticotroph are unknown. For lactotrophs, sommatotrophs and thyrotrophs the pit-1 gene has been identified as an important trans acting factor controlling phenotype, and some progress made towards defining the factors controlling pit-1 expression (Ingraham, Chen et al. 1988; Ingraham, Flynn et al. 1990; Simmons, Voss et al. 1990). The pit-1 protein is a transcription factor that binds to the promoter of the GH and PRL genes, and also to its own promoter (Ingraham, Flynn et al. 1990). During development pit-1 expression becomes activated in response to an as yet uncharacterised stage specific factor and there then follows an induction of pit-1 expression that is

responsible for the differentiation and proliferation of somatotrophs and lactotrophs. In the Snell mouse, which has a mutant Pit-1 gene, the pituitary is hypoplastic and lacks somatotrophs, lactotrophs and thyrotrophs (Li, Crenshaw et al. 1990). The pit-1 promoter is activated by pit-1 and also by growth hormone releasing hormone (GHRH) through cyclic AMP, so that the hypothalamus plays a part in induction of pit-1 expression.

1.5.2 Expression of POMC message and peptide

Measurement of ACTH content of the pituitary is problematic since there is differential post-translational processing of the POMC peptide in the NIL and anterior pituitary. In adult animals, corticotrophs in the anterior pituitary and melanotrophs in the NIL express different prohormone convertase enzymes (Day, Schafer et al. 1992; Zhou, Bloomquist et al. 1993). The principal products of POMC processing in the adult anterior pituitary are ACTH, β-endorphin and β-lipotropin, wheras in the NIL desacetyl α-MSH and β-endorphin (which are further converted to α-MSH and acetyl β-endorphin) predominate (Smyth, Massey et al. 1979; Eipper and Mains 1980; Miller, Johnson et al. 1980; Browne, Bennett et al. 1981; Donohue and Dorsa 1982; Smith and Funder 1988).

It is likely that the anterior pituitary and NIL differentially process the POMC peptide in fetal life. The fetal sheep anterior pituitary contains message and immunoreactive proconvertase 1 activity (which is involved in cleaving POMC to ACTH), but not proconvertase 2 (which further processes ACTH), wheras the fetal NIL expresses both enzymes (Bell, Myers et al. 1998). Following homogenisation of whole fetal pituitary, three large molecular weight ACTH precursors can be separated from the smaller ACTH, β–Melanocyte stimulating hormone (βMSH), β-lipotropin (βLPH), γLPH, and β-endorphin molecules (Silman, Holland et al. 1979). If the NIL is dissected from the anterior pituitary, then only ACTH and the larger POMC fragments are identified (Challis and Brooks 1989). There is evidence that the corticotroph processing of POMC matures through gestation: cultured fetal pituitary cells release increasing amounts of bioactive ACTH, relative to the

larger molecular weight precursors, with increasing gestation (Brieu and Durand 1987).

The expression of POMC message has been examined by Northern analysis and by in-situ hybridisation. Message levels for POMC in the pituitary as measured by Northern blot show either a progressive rise upto day 140 which is maintained in the neonate (Yang, Challis et al. 1991; Myers, Myers et al. 1993) or a fall in late gestation (McMillen, Mercer et al. 1988; Brooks, Currie et al. 1992; Merei, Rao et al. 1993). In-situ hybridisation demonstrates that there is high and increasing expression of POMC in late gestation in the inferior (peripheral) aspect of the anterior pituitary, and lower unchanging expression in the superior aspect of the gland in the region adjacent to the NIL (Matthews, Han et al. 1994). Presumably, the earlier Northern analyses have been confounded by sampling both areas. Expression of POMC message in the NIL is higher than in the anterior pituitary (Matthews, Han et al. 1994). Levels in the NIL reach a plateau at about day 120 and remain high into neonatal life. It is worth noting that measurement of steady state mRNA levels does not necessarily reflect translation to protein, since for many genes there is a considerable amount of post-transcriptional regulation (Day and Tuite 1998).

1.5.3 Circulating ACTH and other POMC peptides

In intact animals mean circulating levels of ACTH start to increase before cortisol (Norman, Lye et al. 1985). The secretion of ACTH in the fetus is pulsatile, at least from day 126 gestation (Brooks and Challis 1991; Apostolakis, Longo et al. 1992; Canny, Young et al. 1998). The presence of pulses of ACTH implies relatively mature neuroendocrine mechanisms controlling ACTH secretion. Recently, however, the persistence of ACTH pulses in hypothalamo-pituitary disconnected fetuses has been demonstrated suggesting that pulsatile secretion is an intrinsic property of the pituitary (Canny, Young et al. 1998). There are no studies that have systematically examined the ontogeny of pulsatile ACTH secretion. In premature human infants sampled on the neonatal unit, deconvolutional analysis suggests the

characteristics of the ACTH pulse change with increasing post-natal age, indicating continued maturation of the pulse generator over this period (Metzger, Wright et al. 1993). The physiological significance of pulsatile ACTH secretion is unclear since the ovine fetal adrenal responds equally well to continuous and pulsatile ACTH administration with no evidence of desensitisation (Lye, Sprague et al. 1983; Jacobs, Young et al. 1994).

With increasing gestation there is a change in the pattern of POMC-derived products present in the circulation. The plasma of the late gestation fetus contains proportionally more low molecular weight, bioactive ACTH relative to higher molecular weight precursors (Brieu and Durand 1987; Saphier, Glynn et al. 1993; Carr, Jacobs et al. 1995). Using specific antibodies directed at epitopes near the carboxy and amnio terminals of the various POMC peptides the relative changes in ACTH and the large N-terminal fragment of POMC, N-POMC $_{(1\text{-}77)}$, in late gestation have been examined (Saphier, Glynn et al. 1993). Concentrations of N-POMC $_{(1\text{-}77)}$ are found to be 20 to 50 times greater than ACTH up to the last week of gestation in the ovine fetus. As concentrations of ACTH increase during the surge in HPA axis activity, there is a fall in the concentration of N-POMC $_{(1\text{-}77)}$ and an increase in gMSH (a product of N-POMC $_{(1\text{-}77)}$ cleavage). The much greater amounts of N-POMC $_{(1\text{-}77)}$ relative to ACTH have led to the suggestion that these alternative POMC gene products arise from the NIL, rather than the anterior pituitary (Saphier, Glynn et al. 1993).

The physiological role of N-POMC $_{(1\text{-}77)}$ is unknown, but N-terminal peptides may play a role in controlling adrenal growth. Adrenal regeneration in adult rats has been shown to be mediated by N-terminal POMC peptides (Estivariz, Morano et al. 1988). Others have demonstrated that ACTH precursors antagonise the response of cultured adrenal cells to ACTH (Schwartz, Kleftogiannis et al. 1995). Studies on the cloned ACTH receptor, however, indicate that the ACTH receptor binds ACTH alone and not other POMC products (kapas, Cammas et al. 1996).

1.5.4 Pituitary responses to hypothalamic releasing factors

The pituitary of ovine fetuses from at least day 70 onwards is capable of responding to both AVP and CRH (MacIsaac, Congiu et al. 1989; McFarlane, Potocnik et al. 1995). In vivo and in vitro both peptides cause a dose dependent increase in ACTH secretion and are found to synergise with each other when co-administered (Norman and Challis 1987; MacIsaac, Congiu et al. 1989). Vasopressin administered in vivo elicits a rapid short term increase in plasma ACTH concentrations wheras CRH causes a slower more sustained increase in ACTH concentration (Pradier, Davicco et al. 1986; Norman and Challis 1987). Some of the differences in ACTH response are probably due to the different circulating half-lives of the two peptides, but there may be separate subpopulations of corticotrophs respond either to CRH or to AVP (Schwartz, Ash et al. 1994).

The relative sensitivity to AVP and CRH seems to change in later gestation. With increasing gestation there is a decline in the ACTH response to challenge with a bolus of AVP (Norman and Challis 1987). In contrast, over the period from 110-130 days the ACTH response to CRH increases (Norman, Lye et al. 1985; Norman and Challis 1987). After 130 days the response to CRH declines somewhat and this has been correlated with a decrease in the number of pituitary binding sites. Down regulation of the receptor and diminished ACTH response may be a consequence of increased CRH secretion at this time, as CRH is known to down-regulate its own receptor (Hauger and Aguilera 1993), or to the negative feedback actions of high concentrations of cortisol.

Attempts to induce parturition in the sheep by administration of CRH or AVP to the fetus have produced conflicting results. Continuous infusion of large amounts of CRH (2.4 μg/h) into the fetal circulation from day 125 result in premature delivery after a period of 5-10 days (Wintour, Bell et al. 1986). In those animals that lambed, however, maternal plasma progesterone concentration did not decrease in the manner normally seen prior to spontaneous term labour (and premature labour induced by ACTH treatment) so that it is difficult to know the physiological relevance of this observation.

Others find that pulsatile administration of CRH (1 μg) or CRH (1 μg) and AVP (200 ng) every 4 h to the fetus over a period of 7 days results in a modest elevation of cortisol but a reduction in the amount of ACTH secreted in response to each bolus of releasing factor (Brooks, Challis et al. 1987; Brooks and White 1990). The net result is that a new steady state of pituitary-adrenal function is achieved which does not result in parturition. A similar effect is seen with chronic exposure to stress and is presumably due to cortisol negative feedback (Gagnon, Challis et al. 1994).

1.5.5 Pituitary CRH and AVP receptors

The action of AVP and CRH at the pituitary is mediated through distinct 7-transmembrane spanning G-protein coupled receptors. The receptor for CRH has been cloned and sequenced in rats and humans, and its distribution determined in adult animals (Chen, Lewis et al. 1993; Perrin, donaldson et al. 1993; Grigoriadis, Lovenberg et al. 1996). There are as yet no reports of message levels in the fetal pituitary of any species. Instead CRH binding capacity has been used to measure receptor number. In the ovine fetus, the CRH binding capacity of the pituitary expressed per unit tissue weight, increases to a maximum at day 130 and then declines at term in a manner very similar to the observed sensitivity to exogenous CRH (Lu, Yang et al. 1990). It is not known if mature and immature corticotrophs express the CRH receptor differently. The decline in CRH binding in late gestation can be explained in terms of receptor down regulation in response to increasing secretion of AVP and CRH (Hauger and Aguilera 1993).

The AVP receptor (V1-b receptor) in the pituitary gland is distinct from renal V2 and vascular and liver V1-a receptors (Arsenijevic, Dubois-Dauphin et al. 1994; Peter, Burbach et al. 1995) There is no information on pituitary AVP binding or receptor expression in the fetal sheep. In models of chronic stress, where concentrations of glucocorticoids are persistently elevated, the pituitary ACTH response to AVP remains refractory to the negative feedback actions of glucocorticoids, in contrast to CRH (Harbuz and Lightman 1992; Aguilera 1994). Paradoxically, glucocorticoid treatment reduces the number

of pituitary V1-b receptors but actually increased the inositol triphosphate response to AVP (Rabadan-Diehl and Aguilera 1998). These observations indicate that the pituitary V1-b receptor may have a role in mediating the late gestation increase in ACTH secretion in the presence of elevated circulating concentrations of cortisol.

1.6 Development of the adrenal gland

1.6.1 Differentiation and maturation of the adrenal cortex

The adrenal gland undergoes marked hypertrophy and hyperplasia in late gestation. The sheep fetal adrenal doubles in weight over the last 12-14 days of gestation, the period of increasing cortisol secretion. This is correlated with the appearance of an adult type glandular histology with definitive zona glomeulosa, fasiculata and reticularis (Robinson, Rowe et al. 1979; Tangalakis, Coghlan et al. 1989). Similarly in the primate (Mesiano, Coulter et al. 1993), cow (Conley, Head et al. 1992) and pig (Conley, Rainey et al. 1994) patterns of enzyme activity and distribution in the fetal adrenal gland only come to resemble the adult late in gestation. Earlier in gestation, between 90 and 120 days of gestation the ability of the fetal ovine adrenal to synthesise cortisol is poor with low levels of expression of steroid synthetic enzymes (Tangalakis, Coghlan et al. 1989). Even earlier in gestation, however, the very immature fetal ovine adrenal possesses the capability for steroid synthesis and is able to respond in vitro to ACTH (Wintour, Brown et al. 1975; Tangalakis, Crawford et al. 1994). A role for fetal adrenal steroids in early gestation has not yet been established and it is not clear if the immature adrenal is functional in vivo.

Early in embryonic development cells of the urogenital ridge destined to become steroid synthesising cells express the nuclear transcription factor steroidogenic factor-1 (SF-1), which binds to the promoter of the cytochrome P_{450} steroid hydroxylases and is present in all steroidogenic cells (Parker and Schimmer 1997). The factors controlling early differentiation and expression of SF-1 are unknown. Further growth and differentiation of adrenal cortical

cells is dependent upon the fetal pituitary. Following hypophysectomy in the ovine fetus or in anencephalic human fetuses, the adrenals remain small (Chez, Hutchinson et al. 1970; Gray and Abramovich 1980).

Much of the pituitary control of adrenal growth seems to be due to ACTH. Premature exposure of the ovine adrenal to continuous or pulsatile ACTH infusion is sufficient to produce hypertrophy, induce enzyme activity and increase cortisol output (Durand, Cathiard et al. 1982; Lye, Sprague et al. 1983). Maternal dexamethasone treatment at around day 50-60 in the sheep results in a decrease in expression of messenger RNA for steroid synthetic enzymes in the fetal adrenal. This has been interpreted as showing that the low levels of ACTH present in the fetal circulation (and which are suppressed by dexamethasone) are important for adrenal development (Tangalakis, Crawford et al. 1994).

The actions of ACTH are probably mediated by locally produced growth factors (Feige, Vilgrain et al. 1998). In the adult animal adrenal cells are renewed from a stem cell pool situated adjacent to the capsule of the gland. As the cells are pushed centripedally towards the medulla, they express the sequentially the enzymes of the glomerulosa, fasiculata and reticularis (Feige, Vilgrain et al. 1998). Similar centripedal migration seems to occur in the developing adrenal gland (Mesiano and Jaffe 1997). Differentiation of cell phenotype is dependent upon ACTH since deletion of POMC expressing cells in adult transgenic mice results in a loss of fasiculata and reticularis cells, while the glomerulosa layer (aldosterone synthesis) remains functional (Allen, Carey et al. 1995). Alternative products of POMC processing have also been identified as candidate trophic factors for the fetal adrenal (Challis and Torosis 1977; Glickman, Carson et al. 1979; Llanos, Ramachandran et al. 1979; Baird, Kan et al. 1983; Saphier, Glynn et al. 1993).

1.6.2 Circulating adrenal steroids

An increase in the concentration of corticosteroid in the fetal sheep circulation late in gestation has been demonstrated by many authors

(Nathanielsz, Comline et al. 1972; Jones, Boddy et al. 1977; Hennessey, Coghlan et al. 1982). Corticosteriods in the fetal circulation arise from the fetal adrenal glands and by transplacental passage from the maternal circulation. Early in gestation the fetal adrenal glands are a minor source of steroid synthesis. Instead corticosteriods are derived largely from the maternal circulation. Fetal adrenalectomy before day 120 makes very little difference to circulating levels of corticosteroids in the fetal sheep (Wintour, Coghlan et al. 1980). Maternal corticosteroid concentrations are about 10 fold greater than fetal concentrations at this time (Hennessey, Coghlan et al. 1982). Fetal cortisol levels increase progressively over the last 15 days or so of gestation so that at term fetal cortisol concentrations are higher than maternal concentrations (Nathanielsz, Comline et al. 1972; Hennessey, Coghlan et al. 1982). Studies infusing tritiated cortisol into the maternal circulation demonstrate that placental transfer of cortisol accounts for all of the cortisol in the fetal circulation before day 120, 37% between days 122 and 135, and from day 135 onwards only 12% or less (Hennessey, Coghlan et al. 1982). This is consistent with an increase in fetal adrenal production of cortisol. After birth levels of cortisol decline (Nathanielsz, Comline et al. 1972) indicating that the increase in cortisol in late gestation is specifically driven in utero. Data in the primate also indicate that fetal corticosteroids are largely of maternal origin until quite late in gestation (Pepe, Waddell et al. 1990).

The ovine fetal adrenal is also a source of androgenic precursors that can be aromatised in the placenta to estrogens. The principal androgen produced by the ovine adrenal is androsteinedione which is metabolised to estradiol in the placenta. Little unconjugated estradiol is present in the circulation until near term when there is a dramatic increase during the preparturient surge in estradiol concentrations (Challis 1971). It is estimated that the ovine fetal adrenal contributes precursors for as much as one third of the estrogen produced during the surge (Mitchell, Lye et al. 1986).

Both maternal and fetal adrenals contribute androgenic precursor for aromatisation in primate pregnancy and this may explain why birth in the primate is not so crucially dependent upon a functioning fetal adrenal. There

are, however, interspecies differences in the contributions of the various precursors to overall estrogen synthesis (Honnebier and Nathanielsz 1994). The human fetal adrenal zone produces mainly dehydroepiandrosterone (DHEA) and 16-hydroxy DHEA from which estradiol and estriol respectively are formed. Nearly 90% of estriol in the maternal circulation is produced from fetal adrenal precursors (Sitteri and MacDonald 1966). By about 35 weeks of gestation there is a dramatic increase in estriol levels paralleling the increase in fetal cortisol levels. It seems likely that both cortisol and androgen are driven by rising ACTH concentrations (Walsh, Norman et al. 1979) but there is debate over the existence of a separate factor capable of influencing androgen secretion independently of cortisol (McKenna and Cunningham 1991)

1.6.3 Adrenal responsiveness to ACTH

In contrast to the declining pituitary response to AVP and CRH in late gestation, the ovine fetal adrenal response to ACTH increases from day 120 onwards (Rose, Meis et al. 1982). Associated with this the number of ACTH binding sites in ovine fetal adrenal membranes are increased (Durand 1979). Pulsatile, or continuous administration of ACTH accelerates the increase in adrenal sensitivity, and results in increasing production of cortisol and parturition (Lye, Sprague et al. 1983; Jacobs, Young et al. 1994). This self-priming action of ACTH on adrenal responsiveness may be mediated by cortisol itself since concurrent treatment with metopirone prevents the increase in fetal adrenal weight stimulated by ACTH infusion (Challis, Huhtanen et al. 1985). Between day 90-120, during the period when the adrenal expression of steroidogenic enzymes is low, the ovine fetal adrenal mounts only a small cortisol response to challenge with ACTH in vitro and in vivo (Rose, Meis et al. 1982).

Other POMC derived peptides are also reported to stimulate adrenal steroid synthesis and secretion. For instance, α-MSH stimulates cortisol secretion from the fetal rabbit and sheep adrenal in vivo (Challis and Torosis 1977; Llanos, Ramachandran et al. 1979) and human adrenal in vitro

(Glickman, Carson et al. 1979; Baird, Kan et al. 1983), though others have been unable to demonstrate any stimulatory effect of α–MSH on a primate species in vivo (Walsh, Norman et al. 1979). Not all of these endogenous opioid-like peptides are acting directly at the adrenal (Bousquet, Lye et al. 1984). The different zonation and steroid products of the primate fetal adrenal have led to speculation that fetal cortisol and androgens may be differentially controlled. In culture α-MSH stimulated DHEA production at lower doses and cortisol at higher doses. The dose of α-MSH used, however, was 10-100 times greater than that of ACTH to elicit the secretion of both hormones (Baird, Kan et al. 1983).

1.7 Cortisol negative feedback

1.7.1 The establishment of negative feedback

Considerable evidence supports the existence of a functioning negative feedback loop from around day 100 upto about day 140 in the ovine fetus. Short term infusion of cortisol or dexamethasone will lower basal levels of ACTH and inhibit the response to CRH and AVP, hypoxaemia or nitroprusside between d110 and d130 gestation (Jones and Ritchie 1977; Wood and Rudolph 1983; Dix, Rose et al. 1984; Rose, Hargrave et al. 1985; Norman and Challis 1987; Ozolins, Young et al. 1990; Wood and Keller-Wood 1991). Conversely adrenalectomy results in elevated ACTH secretion (Wintour, Coghlan et al. 1980; Rose, Turner et al. 1988; McMillen, Antolovich et al. 1990). Associated with the increase in plasma ACTH after adrenalectomy there is approximately a 200% increase in message for CRH in the PVN and POMC message in the anterior pituitary as measured by Northern blot analysis (McMillen, Antolovich et al. 1990; Myers, Ding et al. 1991; Myers, Myers et al. 1993).

1.7.2 Does negative feedback sensitvity decline?

The sustained increase in plasma concentrations of ACTH in the face of elevated levels of corticosteroids in late gestation presents a paradox, since normally corticosteroid negative feedback would be expected to limit the activity of the hypothalamo-pituitary-adrenal axis. Earlier in gestation repeated stimulus to the HPA axis does not lead to sustained increases in plasma cortisol to levels capable of inducing parturition (Brooks, Challis et al. 1987; Brooks and White 1990; Gagnon, Challis et al. 1994). The steady increase in plasma ACTH and cortisol in late gestation suggest that there are specific mechanisms operating to allow the hypothalamus and pituitary to escape from adrenal steroid negative feedback.

Several studies have examined the effects of exogenous cortisol during the last few days of gestation when ACTH and cortisol levels are changing dramatically. One study (Wood 1988) conducted between 138-142 days gestational age concluded that elevation of cortisol to 50-60 ng/ml did not affect basal secretion or the ACTH response to nitroprusside at this gestation. On the basis of these results it has often been accepted that there is a decline in negative feedback sensitivity near term. In contrast, basal ACTH secretion at term (d138-140) is suppressed by cortisol infusion achieving levels of 40-80ng/ml (Ozolins, Young et al. 1990; Wood and Keller-Wood 1991). Others have even found that infusion of cortisol enhances ACTH secretion (Apostolakis, Longo et al. 1994).

One way of rationalising these results is to postulate that the action of cortisol on the fetal HPA axis is akin to the positive feedback actions of estrogen during the luteinising hormone (LH) surge (Moenter, Caraty et al. 1990; Karsch, Bowen et al. 1997; Levine 1997; Spies, Francis Pan et al. 1997). If the analogy is correct then the discrepancy between studies on positive and negative feedback effects of cortisol can be partly explained by methodology: negative feedback effects may be apparent at relatively lower concentrations and shorter exposures to cortisol but elevation above a threshold for a critical period of time is associated with positive feedback to reinforce increasing ACTH secretion.

1.7.3 Anatomic sites of negative feedback

Glucocorticoid receptors are found in multiple sites throughout the HPA axis indicating the potential for feedback directly at the pituitary, hypothalamus or other brain areas important in the regulation of ACTH secretion. Pituitary glucocorticoid receptor concentration measured by triamcinolone binding to dispersed cells is highest at day 110 and then declines threefold through to day 130. By term receptor concentration has risen again though not to the same levels as at day 110 (Yang, Jones et al. 1990). The number of hypothalamic triamcinolone binding sites was always less than that of the pituitary, and after peaking at day 110 declined steadily through to term. The decline in glucocorticoid receptor number is attributed to the down regulation of receptor produced by cortisol itself. In support of this treatment with ACTH at day 125 for 100 hours produced a 30% decline in receptor number (Yang, Jones et al. 1990). In contrast to studies measuring receptor binding sites, Northern analysis reveals message for glucocorticoid receptor is present in the hypothalamus, pituitary and adrenal from day 60 to term in unchanging concentrations (Yang, Hammond et al. 1992). A more recent analysis of message for the glucocorticoid receptor by in situ hybridisation finds that the level of message in the pituitary gland increases markedly with the transition into active labour (Matthews, Yang et al. 1995).

The relative sensitivity of pituitary and suprapituitary feedback pathways and their development during fetal life is poorly characterised. Corticosteroids have a direct action on the pituitary gland to suppress ACTH secretion. Treatment with cortisol or dexamethasone inhibits the ability of CRH and AVP to stimulate ACTH secretion from at least day 120 onwards, but crucially, pituitary sensitivity during the preparturient surge in ACTH and cortisol has not been examined (Rose, Hargrave et al. 1985; Norman and Challis 1987; Ozolins, Young et al. 1990). Studies on suprapituitary sensitivity to feedback have produced conflicting results. Evidence from the hypothalamo-pituitary disconnected preparation indicates that corticosteroid suppression of basal ACTH secretion near term (138 days) requires an intact hypothalamo-pituitary axis, but that before 130 days gestation basal ACTH

secretion is not affected by a similar levels of corticosteroids in either intact or hypothalamo-pituitary disconnected fetuses (Ozolins, Young et al. 1990). In other words suprapituitary feedback does not seem to be important before day 130 gestation. Others, however, find that around day 125 gestation placement of dexamethasone implants bilaterally adjacent to the PVN decreases CRH and AVP immunostaining within the PVN and prevents the ACTH response to stress (McDonald, Hoffmann et al. 1990). The same treatment also reduces CRH message content of PVN micropunches as measured by Northern analysis but not pituitary POMC message content (Myers, McDonald et al. 1992). Plasma concentrations of ACTH are similarly unaffected by dexamethasone implants (McDonald, Hoffmann et al. 1990). The degree to which CRH message in the micropunches is depressed by the dexamethasone implants decreases nearer term consistent with a decline in negative feedback sensitivity.

1.7.4 Corticosteroid binding globulin

Circulating corticosteroid is largely bound to corticosteroid-binding globulin (CBG). In vitro experiments demonstrate that CBG serves to sequester free active corticosteroid and reduce its biological potency (Berdusco, Yang et al. 1995), though others have argued that some cells may actually possess receptors for unliganded CBG (Maitra, Khan et al. 1993). An increase in CBG offers a mechanism for overcoming feedback by reducing the amount of free bioactive steroid. Although concentrations of CBG increase in the fetal circulation throughout gestation, levels of free cortisol also increase (Fairclough and Liggins 1975; Ballard, Kitterman et al. 1982). The main site of CBG production is the fetal liver (Seralini, Smith et al. 1990). There is evidence that cortisol itself can stimulate hepatic synthesis of CBG in the fetal sheep (Challis, Nancekievill et al. 1985; Berdusco, Hammond et al. 1993; Berdusco, Milne et al. 1994). The fetal pituitary expresses the gene for CBG adding the possibility of paracrine mechanisms that overcome the negative feedback action of cortisol (Berdusco, Yang et al. 1995).

1.7.5 11β hydroxysteroid dehydrogenase

Access to intracellular glucocorticoid receptors can be controlled by cytoplasmic enzyme activity. The enzyme 11β hydroxysteroid dehydrogenase (11βHSD) converts the biologically active steroids cortisol and corticosterone to the inactive metabolites cortisone and 11-dehydrocorticosterone respectively. There are at least two isoenzymes with different tissue distributions and different functions (Steward, Whorwood et al. 1995; Gomez-Sanchez and Gomez-Sanchez 1997; Penning 1997). An NADP+ dependent enzyme (11βHSD-1) is present predominantly in liver and proximal collecting tubules of the kidney. The enzyme appears to be bidirectional and has a Km for corticosterone of 2 μM. The tissue distribution, bidirectionality and low affinity for corticosterone of this isoform suggest that it is not involved in protecting mineralocorticoid and glucocorticoid receptors from corticosteroids. Under physiological conditions 11βHSD-1 may function as a oxo-reductase to increase local cellular concentrations of active steroid (Duperrex, Kenouch et al. 1993; Gomez-Sanchez and Gomez-Sanchez 1997). In contrast, 11βHSD-2 is unidirectional, uses NAD+ as a cofactor, has a Km for corticosterone of 30-50 nM and colocalises with the mineralocorticoid receptor in the rat kidney (Penning 1997). Both isoforms can be inhibited by progesterone metabolites (Souness, Latif et al. 1995).

The sensitivity of the HPA axis to corticosteroid feedback can potentially be modulated by 11βHSD activity. The enzyme is present in the pituitary, PVN and limbic areas in adult rats (Moisan, Seckl et al. 1990; Lakshsmi, McEwen et al. 1991; Seckl, Dow et al. 1993) and treatment with an inhibitor of 11βHSD results in decreased release of CRH and increased release of AVP into portal blood (Seckl, Dow et al. 1993). The concentration of circulating corticosteroid in these experiments, however, remained unchanged despite inhibition of 11βHSD activity. In the fetal sheep message for 11βHSD-1 is present in the hypothalamus and pituitary, and levels of

expression and enzyme activity in the anterior pituitary are increased at term (Yang, Smith et al. 1992; Yang, Matthews et al. 1995). In the sheep 11βHSD-1 seems to act predominantly as a dehydrogenase and so can prevent corticosteroids from gaining access to the glucocorticoid receptor (Yang 1997). Critical studies such as inhibition of 11βHSD activity in the late gestation fetal sheep have not been conducted.

Besides the HPA axis, 11βHSD is present in other steroid sensitive tissue including the placenta and fetal kidney, lungs, gonads, liver and colon (Lopez-Bernal, Flint et al. 1980; Yang, Smith et al. 1992; Brown, Chapman et al. 1993; Albiston, Obeyesekere et al. 1994; Stewart, Murry et al. 1994). Both 11βHSD-1 and 11βHSD-2 are present in human placenta (Sun, Yang et al. 1998). Placental 11βHSD is likely to be important for controlling transplacental passage of cortisol as well as the actions of cortisol within the placenta itself (Sun, Yang et al. 1998). It has been suggested that 11βHSD additionally protects progesterone or androgen regulated genes from activation by corticosteroids (Funder 1994).

In baboons placental NAD+ dependent 11βHSD activity is estrogen regulated (Pepe and Albrecht 1998). Infusion of estrogenic precursor to the mother at d100 gestation (term=184) increases placental 11βHSD activity wheras fetectomy (which removes the source of endogenous estrogenic precursor) decreases activity (Baggia, Albrecht et al. 1990). Functionally, it is argued, the action of estrogen is to lower the circulating cortisol concentration, which at this time in gestation is largely of maternal origin (Pepe, Waddell et al. 1990), and so allow activation of the fetal HPA axis, as inferred by higher levels of expression of POMC message in the fetal pituitary (Pepe, Davies et al. 1994). It is difficult to reconcile the fact that cortisol in these mid-gestation estrogen treated fetuses was not significantly lower than controls and yet still POMC expression was enhanced. Another interpretation is that estrogen directly activates the fetal HPA axis, perhaps by inducing brain and/or pituitary 11βHSD.

1.7.6 Transcription factors

Glucocorticoids regulate the activity of specific genes in a positive or negative manner through a complex nuclear pathway involving glucocorticoid receptors and other nuclear proteins (Horwitz, Jackson et al. 1996; Reichardt and Schutz 1998). The sensitivity to glucocorticoids can be modulated by alterations in the concentrations or biological activity of these nuclear proteins. Positive actions mediated by the glucocorticoid receptor require the formation of a homodimer with a second glucocorticoid receptor before binding to the glucocorticoid response element in the upstream promoter sequence of the gene in question (Reichardt and Schutz 1998). Full activity of the transcription complex then requires the participation of steroid coactivator proteins (Horwitz, Jackson et al. 1996).

Many genes that are inhibited by glucocorticoids do not have a classical glucocorticoid response element, and a number of mechanisms of gene repression by glucocorticoids have been described, involving steric hindrance by the glucocorticoid receptor bound to DNA preventing the binding of other transcription factors, direct interactions between the glucocorticoid receptor and other proteins required for gene activation and the induction of inhibitor proteins (Bamberger, schulte et al. 1996; Cato and Wade 1996; McEwan, Wright et al. 1996; Wissink, Van Heerde et al. 1998). For example, *in vitro* the negative regulation of the naked POMC gene sequence involves binding of three molecules of glucocorticoid receptor to a site in upstream promoter sequence of the POMC gene. The glucocorticoid receptors then interact with unknown transcription factors or components of the transcription activation complex binding to nearby sites in the promoter sequence to interfere with their normal function (Riegel, Lu et al. 1991).

In another model of negative glucocorticoid action, the interaction between the glucocorticoid factor and the transcription factor, activator protein-1 (AP-1) which is itself a dimer, can either enhance or repress gene activity depending upon the particular subunits making up AP-1. The mouse proliferin gene promoter binds both AP-1 and the glucocorticoid receptor. If the AP-1 is a heterodimer of c-jun/c-fos, then the glucocorticoid receptor

represses gene transcription. On the other hand, if the AP-1 is a heterodimer of c-jun/Fra-1, then the glucocorticoid receptor enhances transcription (Cato and Wade 1996). Changes in the ratio of Fos and Fra-1 might then be expected to alter the effects of glucocorticoids on expression of particular genes. It is not known whether such mechanisms are operational in the fetal HPA axis, but it is worth noting that the expression of Fos in the ovine fetal hypothalamus is increased in late gestation (Hoffman, McDonald et al. 1991).

Others have identified a negative glucocorticoid response element that binds the glucocorticoid receptor *in vitro* and inhibits expression of the POMC gene (Drouin, Sun et al. 1993). The same negative response element binds Nur77, an orphan steroid receptor with as yet no known ligand (Okabe, Takayangi et al. 1998), and in a mouse pituitary cell line (AT20) stably expressing the human homologue of Nur77 there is a reduction in dexamethasone suppression of POMC synthesis and ACTH secretion (Okabe, Takayangi et al. 1998). The expression of native Nur77 in AT20 cells is increased by exposure to CRH (Okabe, Takayangi et al. 1998). Thus in the context of chronic stress, the expression of Nur77 in corticotrophs is increased and this acts to reduce glucocorticoid negative feedback.

Finally, the anti-inflammatory actions of glucocorticoids seem to be mediated partly through protein-protein interactions between glucocorticoid receptors and NF-κB, and partly through induction of an inhibitor protein IκB, which prevents NF-κB from being transcriptionally active (Wissink, Van Heerde et al. 1998).

1.8 Peripheral signals to the fetal HPA axis

1.8.1 Metabolic factors

The HPA axis is important not only for the response to stressful stimuli, but also for the maintenance of internal homeostasis. The HPA axis is active during the metabolic transition between the fed and fasted states (Dallman, Strack et al. 1993), and in turn metabolic signals influence the HPA axis. The role of the HPA axis in regulating fetal metabolism has not been investigated.

Metabolic substrates available to the fetus by passive transfer across the placenta fluctuate in concert with levels present in the maternal blood, and even for actively transported substances fetal plasma concentrations are ultimately dependent upon the availability of the metabolite in the maternal plasma (Hay 1995). The fetus is uniquely dependent upon the maternal organism for provision of oxygen and metabolic substrate, but it does seem to be able to modify maternal metabolism through placental hormones secreted into the maternal circulation (Petraglia 1991).

As the fetus grows there is an increasing requirement for oxygen and metabolic substrate to maintain growth and it has been hypothesised that fetal demands overtake the capacity of placental transfer and maternal metabolism to sustain delivery of carbohydrates, amino acids and fatty acids in late gestation (Schneider 1996). Fetal growth in late gestation is rapid: the ovine fetus grows at a rate of approximately 1% bodyweight/day (Fowden 1995) and this imposes high demands for metabolic substrate. Oxygen and glucose uptake by the near term fetal sheep are calculated at 315 and 30 μmol/min/kg respectively (Fowden 1995). A teleological argument could be made that the fetus initiates parturition to escape from a hostile in utero environment. Some evidence supports the idea that metabolic factors trigger birth. Fasting of sheep and humans late in gestation results in premature delivery (Alexander 1956; Kaplan, Eidelman et al. 1983). In human pregnancy, low pre-pregnancy maternal weight, poor weight gain and fetal growth arrest, have been associated with spontaneous premature delivery (Arias, Rodriquez et al. 1993; Hediger, Scholl et al. 1995; Carmichael and Abrams 1997). The contribution of metabolic signals to activation of the fetal HPA axis and the initiation of parturition has not been systematically investigated.

Mild insulin-induced hypoglycaemia is known to be a stimulus for increased ACTH secretion in adult animals (Caraty, Grino et al. 1990; Dohanics, Hoffman et al. 1991) and long term restriction of food intake elevates ACTH and cortisol in all species that have been studied (Dallman, Strack et al. 1993). The hypothalamus in vitro can increase the release of CRH into the bathing media in response to lowering concentrations of glucose

in the extracellular fluid (Widmaier, Plotsky et al. 1988). Under normal physiological conditions hypoglycaemia is accompanied by hypoinsulinaemia, and a fall in plasma insulin concentrations will also stimulate ACTH secretion through a pathway involving insulin receptors in the arcuate nucleus (Schwartz, Figlewicz et al. 1992; Dallman, Strack et al. 1993). Similarly, acute hypoxaemia is a stimulus for ACTH and cortisol secretion (Braems, Matthews et al. 1996). When hypoxia is maintained, plasma ACTH levels return to normal, but anterior pituitary expression of POMC is increased, and plasma cortisol levels remain elevated (Braems, Matthews et al. 1996).

1.8.2 Prostaglandin E_2

The suggestion has been made that increasing placental prostaglandin E_2 (PGE_2) production provides the drive to the fetal HPA axis leading to labour (Thorburn 1991). The placenta and fetal membranes of all species synthesise PGE_2. In the ovine fetus circulating PGE_2 metabolites are found in increasing concentrations throughout gestation and rise still further in labour (Challis, Dilley et al. 1976; MacKenzie, MacLean et al. 1980; Thorburn 1991). Prostaglandin E_2 sensitises all components of the HPA axis: increasing adrenal sensitivity to ACTH, synergising with AVP to promote secretion of bioactive ACTH (Brooks and Gibson 1992; Hollingworth, Deayton et al. 1995), and promoting the release of hypothalamic factors when given intracerebroventricularly (Brooks 1992). Placental synthesis of PGE_2 appears to be part of a stress adaptive pathway: the concentration of PGE_2 metabolite in fetal plasma is increased in response to maternal hypoxia (Hooper, Harding et al. 1992), fetal intravascular volume expansion (Weiner and Robillard 1989) and maternal fasting (Fowden, Harding et al. 1987).

1.8.3 Corticotrophin releasing hormone

In primates trophoblasts of the placenta and fetal membranes express the genes for CRH and for POMC (Cheng, Chang et al. 1986; Riley and Challis 1991; Wu, Unno et al. 1995) and a placental-fetal HPA axis interaction

culminating in parturition has been proposed (Challis and Brooks 1989; Karalis, Goodwin et al. 1996). Placental production of CRH is stimulated by corticosteroids both *in vitro* and *in vivo* (Robinson, Emanuel et al. 1988; Korebritis, Yu et al. 1998; Marinioni, Korebritis et al. 1998) creating a potential positive feedback cascade whereby corticosteroids stimulate placental CRH production, which then stimulates the maternal and fetal pituitary-adrenal axis to secrete more cortisol which in turn promotes further CRH synthesis (Challis and Brooks 1989; Karalis, Goodwin et al. 1996). As a consequence of increasing CRH secretion the fetal and maternal adrenals are also stimulated to secrete precursors for placental estrogen synthesis. Rising concentrations of estrogens in turn result in labour (Mecenas, Giussani et al. 1996) and eventual escape from this positive feedforward cascade.

There is reasonable evidence in primates that placental CRH does have some role in the control of parturition. In human pregnancy maternal plasma concentrations rise to high levels (1300-1700 pg/ml) comparable to those estimated to be present in rat portal plasma (Linton, Wolfe et al. 1991). Interestingly, high frequency blood sampling shows that there are approximately hourly pulses of CRH in maternal plasma with an amplitude of 250pg/ml (Petraglia, Genazzani et al. 1994). Much of the CRH in maternal plasma is bound to a specifc CRH-binding protein which is also produced by the placenta (Petraglia, Potter et al. 1993). From about 35 weeks gestation onwards, however, the concentration of the binding protein in the plasma falls, and concentrations of CRH increase (McLean, Bisits et al. 1995). It has been demonstrated that women destined to labour prematurely have persistently elevated plasma concentrations of CRH compared to gestation matched controls labouring at term, and that women who do not labour until after 42 weeks have persistently lower circulating CRH concentrations (McLean, Bisits et al. 1995). Concentrations of CRH in cord plasma at term are about 2-10-fold lower than those in maternal plasma (Goland, Wardlaw et al. 1986; Economides, Linton et al. 1987; Linton, Wolfe et al. 1991).

The human placenta also synthesises ACTH, and a paracrine pathway regulating placental ACTH secretion is suggested since CRH stimulates the release of ACTH from perifused human placentae in a dose dependent

manner (Margioris, Grino et al. 1988). The placental contribution to the concentration of ACTH in the maternal and fetal circulations is unknown. Associated with increased CRH concentrations in human pregnancy, there is a modest elevation of maternal plasma ACTH (to the upper end of the normal range) and plasma free cortisol, and increased 24hr excretion of urinary free cortisol as pregnancy advances (Linton, Wolfe et al. 1991; Goland, Conwell et al. 1992). Unfortunately, studies in humans and another primate species have shown that a bolus injection of CRH into the maternal circulation fails to elevate ACTH concentrations (Goland, Stark et al. 1990; Schulte, Weisner et al. 1990). This seems to indicate that placental CRH and ACTH secreted into the circulation do not have a major effect on maternal or fetal HPA axes. It is possible that CRH facilitates labour through other mechanisms: the placenta and myometrium expresses CRH receptors (Hillhouse, Grammatopoulos et al. 1993; Clifton, Owens et al. 1995; Wu, Unno et al. 1995; Grammatopoulos, Dai et al. 1998) and CRH synergises with oxytocin to stimulate the myometrium, at least in vitro (Quartero and Fry 1989).

There are considerable species differences in the concentrations of CRH in maternal plasma during pregnancy. In the sheep CRH only becomes detectable in maternal plasma very late in gestation and the concentrations are comparatively low (20-50pg/ml). (Jones, Gu et al. 1989; Wood and Keller-Wood 1991). The ovine placenta does not appear to synthesise or secrete ACTH in vivo (McMillen, Antolovich et al. 1990; Keller-Wood and Wood 1991).

1.8.4 Placental steroids

Estrogen can stimulate the fetal and maternal HPA axis. In adult animals there is a well documented sex difference in resting HPA activity with females having higher resting plasma ACTH and corticosteroid concentrations. In female rats the ACTH and cortisol responses to stress are estrous cycle dependent (Viau and Meaney 1991; Carey, Deterd et al. 1995). Replacement of estrogen in ovariectomised animals results in enhanced secretion of ACTH and cortisol in response to stress (Viau and Meaney 1991;

Burgess and Handa 1992; Carey, Deterd et al. 1995). The mechanism whereby estrogen enhances ACTH and cortisol secretion is not fully understood. In female rats estrogen inhibits corticosteroid induced down-regulation of the hippocampal glucocorticoid receptor (Burgess and Handa 1992). The receptor is still present in unchanged concentration but somehow its efficacy is reduced. At a molecular level the 5'-upstream sequences of the CRH and POMC genes contain potential progesterone/glucocorticoid and estrogen response elements (Lundblad and Roberts 1988; Vamvakopoulos and Chrousos 1994).

The action of estrogen on the fetal HPA axis has been examined in primates and sheep. In the baboon, infusion of androgenic precursors for placental estrogen synthesis into the maternal circulation increases POMC expression in the fetal pituitary (Pepe, Davies et al. 1994). Estradiol produced in the placenta from these androgenic precursors is postulated to contribute to increasing ACTH synthesis by increasing placental 11βHSD production, which in turn removes cortisol from the fetal circulation (Pepe, Davies et al. 1994; Pepe and Albrecht 1998). In contrast, in the sheep fetus the potential for estradiol to stimulate the fetal HPA axis has been investigated in several studies with conflicting results despite all three studies using similar doses of estrogen (Saoud and Wood 1997; Wang, Matthews et al. 1997; Wood and Saoud 1997).

1.9 Hypothalamic pathways regulating ACTH secretion in utero

The hypothalamic pathways regulating HPA activity in the fetus remain little investigated. Inference from the adult is not always satisfactory since it is not known when many of the neurotransmitter pathways and their receptor systems mature. Additionally, receptors for various neurotransmitters often display developmentally regulated patterns of expression and phosphorylation that mean that their functions are subtly different during fetal and neonatal life (Lanius, Pasqualotto et al. 1993). In adult animals a number of pathways mediating the stress response and the diurnal rhythm in HPA activity have been partially characterised pharmacologically and neuroanatomically

(Harbuz and Lightman 1992; Antoni 1993; Dallman, Strack et al. 1993; Whitnall 1993; Chrousos 1995). The following sections briefly review the neuroanatomy of the PVN and those neurotransmitters for which there is experimental evidence indicating a role in regulating the HPA axis in fetal life. In addition the potential role of Neuropeptide-Y in mediating the fetal HPA axis response to metabolic challenge is also considered.

1.9.1 Structure of the paraventricular nucleus

The PVN is a complex structure integrating endocrine and autonomic responses to the environment (Swanson and Sawchenko 1980). Probably most is known about the organisation of the PVN from study of the laboratory rat. In this species, several subdivisions of the nucleus can be recognised. Parvocellular CRH containing neurons projecting to the median eminence are contained in the dorsal medial parvocellular subdivision (Whitnall 1993). About 50% of these neurons co-express AVP. After adrenalectomy nearly all of these neurons express AVP (Whitnall 1988), but generally it seems likely that there are two separate populations of neurosecretory neurons with different locations within the dorsal medial subdivision of the nucleus, that receive different innervation and presumably have different roles in the regulation of ACTH secretion (Whitnall 1993). Magnocellular AVP containing neurons of the SON and PVN projecting to the posterior pituitary may also secrete AVP at the median eminence "en passant" (Antoni 1993).

Lesion studies, electrical stimulation, immunocytochemistry and tract tracing studies have defined a number of brain regions projecting to, and capable of influencing CRH and AVP neurons in the PVN. The hippocampus and amygdala mediate corticosteroid negative feedback, but probably do so by innervating interneurons adjacent to the PVN and in the bed nucleus of the stria terminalis and septal areas (Jacobson and Sapolsky 1991). The suprachiasmatic nucleus, important as the circadian pacemaker, projects to the PVN and drives the diurnal rhythm in HPA axis activity (Cascio, Shinsako et al. 1987). Ascending adrenergic and noradrenergic pathways from the brain stem are activated during stress (Ceccatelli, Villar et al. 1989) and

provide stimulatory input to CRH and AVP containing neurons (Sawchenko 1988; Plotsky, Cunningham et al. 1989). Some of these catecholamine containing neurons also express Neuropeptide-Y (NPY), but the largest projection of NPY immunoreactive fibres arises from the hypothalamic arcuate nucleus (Bai, Yamano et al. 1985). Midbrain serotonergic neurons also provide stimulatory input to the PVN (Whitnall 1993).

1.9.2 N-methyl-D-aspartate receptor coupled pathways

Excitatory amino acid transmitters are widely distributed in the mammalian brain and are important for synaptic plasticity in both developing and adult brain (McDonald and Johnston 1990; Ruzika and Jhamander 1993). As such they might be expected to be important in the developing HPA axis. Pharmacologically, two distinct classes of ion channel coupled receptor are identified, based upon response to the agonist N-methyl-D-aspartate (NMDA): 1/. the NMDA receptor and 2/. the non-NMDA receptor. Molecular characterisation of the NMDA receptor reveals that there are multiple subunits involved in the formation of a receptor complex (Barnes and Henley 1992; Monyer, Sprengel et al. 1992; Hollmann and Heinemann 1994; Seeburg, Burnashev et al. 1995). A common NMDAR1 subunit is found in all functional receptors. At least four NMDAR2 subunits have been identified by screening brain derived cDNA libraries for conserved sequences found in glutamate-operated ion channels (Seeburg, Burnashev et al. 1995). Expression of NMDAR1 with different NMDAR2 subunits in *Xenopus* oocytes results in functional receptors with differing kinetics (Seeburg, Burnashev et al. 1995). The NMDAR1 subunit is ubiquitously expressed throughout the central nervous system, but the expression of the other subunits varies in different brain regions as well as during development (Farrant, Feldmeyer et al. 1994; Monyer, Burnashev et al. 1994; Sheng, Cummings et al. 1994; Seeburg, Burnashev et al. 1995).

A considerable body of evidence indicates that NMDA receptors are involved in neuroendocrine control of ACTH secretion in the adult animal (Brann and Mahesh 1994; Brann 1995; Brann and Mahesh 1997). Receptors

have been localised in the hypothalamus by radioligand binding, by in situ hybridisation studies and by immunocytochemistry (Monyer, Sprengel et al. 1992; Meeker, Greenwood et al. 1994; Urbanski, Fahy et al. 1994; Bhat, Mahesh et al. 1995). Nerve fibres containing glutamate or aspartate, putative excitatory amino acid transmitters, are found in most hypothalamic nuclei including the parvocellular PVN, the SON and the arcuate nucleus (Van den Pol, Waurin et al. 1990; Van den Pol 1991; Goldsmith, Thind et al. 1994; Thind and Goldsmith 1995). Administration of NMDA stimulates ACTH or cortisol secretion in adult rats, primates and sheep (Gay and Plant 1987; Reyes, Luckhaus et al. 1990; Farah, Rao et al. 1991; Chautard, Boudouresque et al. 1993; Brooks and Howe 1996). One study has demonstrated that NMDA stimulates ACTH secretion in the ovine fetus (Brooks and Howe 1996).

The precise site at which NMDA acts to stimulate ACTH secretion is unknown. Although NMDA receptors are found in the pituitary (Meeker, Greenwood et al. 1994; Bhat, Mahesh et al. 1995) it is thought the neuroendocrine effects of NMDA are mediated through supra-pituitary mechanisms. In the case of NMDA stimulated LH secretion there is good evidence for a suprapituitary site of action (Urbanski, Kohama et al. 1996). Microdialysis studies reveal an increase in glutamate release in the preoptic area during the preovulatory LH surge in the ovariectomised steroid primed female rat (Jarry, Leonhardt et al. 1995). Direct sampling of portal plasma by push-pull perfusion in the pre- and peri-pubertal female rhesus macaque shows that NMDA stimulates the release of luteinizing hormone releasing hormone (LHRH) (Claypool and Terasawa 1989). Furthermore the stimulatory effect of NMDA on LH secretion is blocked by pretreatment with an LHRH receptor antagonist (Plant, Gray et al. 1989). Limited evidence also supports a suprapituitary site of action to stimulate ACTH secretion. The relatively rapid ACTH response to NMDA suggests that the action is likely to involve direct stimulation of AVP and CRH neurons in the PVN or closely associated interneurons. One study has demonstrated *in vivo* that injection of glutamate into the amygdala increases median eminence CRH release (Gabr, Birkle et al. 1995), whilst *in vitro* NMDA stimulates the release of both AVP

and CRH from hypothalamic explants and slices (Costa, Yasin et al. 1992; Patchev, Karalis et al. 1994; Joanny, Steinberg et al. 1997). Recently, however, it has been demonstrated that the ACTH response to NMDA in the ovine fetus cannot be blocked with AVP or CRH antagonists (Szeto, Soong et al. 1999).

The role of endogenous excitatory amino acids in the regulation of HPA axis activity during fetal life has not been characterised. In the ovine fetus, the ACTH response to a bolus of NMDA increases with gestation, compatible with a role for excitatory amino acids in driving the late gestation increase in HPA axis activity (Brooks and Howe 1996). It should be remembered, however, that this does not necessarily convincingly demonstrate that the endogenous ligand(s) acting at the NMDA receptor regulate ACTH secretion. Furthermore, there are no reported studies on the ontogeny of NMDA receptor distribution, binding or expression in the neuroendocrine hypothalamus.

1.9.3 Neuropeptide-Y and the arcuate nucleus

Neuropeptide-Y is 36 amino acid peptide neurotransmitter found in several brain regions including the hypothalamus where it is localised most intensely in cell bodies in the arcuate nucleus (Leibowitz 1991; Kalra and Crowley 1992). From the arcuate nucleus there is a strong projection to the PVN where NPY-immunoreactive fibres are seen to contact CRH neurons (Bai, Yamano et al. 1985; Liposits, Sievers et al. 1988). Ascending noradrenergic and adrenergic fibres also contain NPY and after transection of these pathways there is an appreciable decline in hypothalamic NPY content. These hypothalamic and extrahypothalamic pathways are able to modulate pituitary-adrenal function. For example, injection of NPY directly into the PVN or infusion via the third cerebral ventricle stimulates ACTH and corticosteroid secretion in the dog, rat and adult sheep (Wahlestedt, Skagerberg et al. 1987; Inoue, Inui et al. 1989; Porter, Naylor et al. 1993; Zarjevski, Cusin et al. 1993) and immunoneutralisation of central NPY prevents the ACTH and cortisol response to insulin-induced hypoglycaemia (Inui, Inoue et al. 1990). At a

hypothalamic level NPY stimulates CRH synthesis and release (Haas and george 1987; Haas and George 1989).

Many of the functions of NPY in the hypothalamus are concerned with co-ordinating the behavioural and endocrine responses to energy deprivation (Leibowitz 1991; Friedman and Halaas 1998). Levels of NPY message in the arcuate nucleus are increased after fasting and decreased in the fed state indicating that NPY expression is regulated by metabolic state (Brady, Smith et al. 1990; Adam, Findlay et al. 1997).

Metabolic feedback onto hypothalamic NPY systems is provided by several peptides present in the peripheral circulation. Circulating insulin and insulin-like growth factor concentrations are acutely elevated in the fed state in adult animals (Dallman, Strack et al. 1993; Thissens, Ketelslegers et al. 1994) while the adipocyte product leptin signals the longer term state of the body fat stores (Zhang, Proenca et al. 1994; Bennett 1995; Lindpainter 1995; Bray 1996; Wurtman 1996). The well nourished animal has high circulating leptin, insulin and insulin growth factor concentrations. These peripheral hormones are able to reach the arcuate nucleus because of a deficiency in the blood brain barrier at this site, or are specifically transported into the cerebrospinal fluid by the choroid plexus (Schwartz, Figlewicz et al. 1992; Reinhardt and Bondy 1994; Caro, Kolaczynski et al. 1996; Schwartz, Peskind et al. 1996).

Intracerebroventricular administration of insulin decreases NPY message expression in the arcuate nucleus (Schwartz, Sipols et al. 1992). Similarly, leptin decreases NPY synthesis (Stephens, Basinski et al. 1995). As a result arcuate drive to the PVN is reduced, and circulating ACTH and cortisol concentrations fall. In the transition to the fasting state, insulin and leptin concentrations are lowered, and the NPY system is disinhibited so increasing HPA axis activity. In turn corticosteroids increase tissue insulin resistance and stimulate insulin and leptin synthesis (Saladin, De Vos et al. 1996). Thus there is an endocrine loop regulating body mass and energy balance that involves NPY neurons in the arcuate nucleus, the parvocellular PVN, and peripheral corticosteroids, insulin and leptin.

There are no studies that have examined the ontogeny of the fetal HPA axis response to NPY in any species. Certainly, there is expression of NPY message in the mediobasal hypothalamus of the fetal sheep from at least day 110 onwards as measured by in situ hybridisation, and the expression of message increases with gestation (Warnes, Morris et al. 1998). Maternal undernutrition or cortisol infusion increased NPY gene expression and the authors speculate that metabolic cues may participate in initiating parturition (Warnes, Morris et al. 1998). Since NPY containing neurons are involved in pathways other than those regulating HPA axis activity, it remains possible that the increase in mediobasal hypothalamic NPY gene expression with increasing gestation is not coupled to HPA axis activity. Studies administering NPY in to the fetal hypothalamus are required to establish functionality.

1.9.4 Opiates

Opioids function generally to pre-synaptically inhibit neurotransmitter release, and to generate inhibitory post-synaptic potentials. Several hypothalamic nuclei either contain opioidergic neurons or receive opioid projections. The rat PVN receives a projection from the arcuate nucleus of fibres that are immunoreactive for ACTH, α-MSH and β-endorphin (Akil, Watson et al. 1984; Khachaturian, Lewis et al. 1985). In situ hybridisation studies in the adult sheep brain find POMC message in the arcuate nucleus and pre-proenkephalin in the PVN (Whisant, Curto et al. 1992; Broad, Kendrick et al. 1993). Opioid receptors are present in the fetal sheep hypothalamus in late gestation, but not in the pituitary (Yang and Challis 1991). The effect of opioid receptor activation is likely to be complex, since it is possible for opioids to have a stimulatory action through disinhibition of inhibitory pathways, to presynaptically inhibit excitatory pathways and to be directly inhibitory to post-synaptic neurons. Opioid pathways are active in the ovine fetus in late gestation since intravenous infusion of the mu receptor antagonist naloxone during the pre-parturient surge in ACTH secretion decreases circulating ACTH concentrations (Brooks and Challis 1991). In

fact from about day 125 gestation, a specific Met-enkephalin analogue will stimulate ACTH and cortisol secretion and this effect is antagonised by naloxone (Brooks and Challis 1988). In contrast to the effects of opioids during the increase in ACTH secretion at term, naloxone has no effect on the ACTH response to acute intra-uterine hypoxia (Brooks and Challis 1992). Others report that the kappa agonist dynorphin A1-13 given systemically stimulates ACTH secretion in the ovine fetus, and since the response is not attenuated by CRH or AVP antagonists, suggest a pituitary site of action (Szeto, Soong et al. 1999).

1.9.5 Endothelin

Several central peptidergic neurotransmitters may modulate HPA axis activity, but there is little information on the ontogeny and activity of such pathways during fetal life. Members of the endothelin family of peptides and their receptors have been localised by immunocytochemistry and in situ hybridisation to the PVN and other hypothalamic sites in adult human brains (Takahashi, Ghatei et al. 1991). The actions of endothelin-3 in the ovine fetus have been examined. Both central and peripheral administration of endothelin-3 stimulate ACTH and cortisol secretion, though peripheral administration of endothelin-3 was associated with transient hypoxia and acidosis that may have provided a non-specific stimulus to ACTH secretion (Greer, Thomas et al. 1995).

1.9.6 Dopamine

A number of studies have examined the effect of dopamine agonists and antagonists on the secretion of POMC derived peptides in the ovine fetus in an attempt to dissect out the relative contributions of the NIL and anterior pituitary to circulating ACTH concentrations. The evidence is conflicting. Intravenous infusion of bromocriptine, a specific D2 receptor agonist, decreases the expression of POMC in the NIL but not in the anterior pituitary (Matthews, Fraser et al. 1996). Measurement of circulating hormone

concentrations supports the contention that dopamine regulates the NIL but not the anterior pituitary since bromocriptine decreased plasma α-MSH concentrations, but was without effect on plasma ACTH(1-39) or cortisol concentrations (Hagan and Brooks 1996; Matthews, Fraser et al. 1996). Basal secretion from the NIL is under endogenous inhibitory dopaminergic tone as the dopamine antagonists sulpiride and metoclopramide significantly increased plasma α-MSH concentrations (Newman, Wardlaw et al. 1987; Hagan and Brooks 1996). Antagonism of endogenous dopaminergic pathways also acutely increases plasma ACTH concentrations, however, in contrast to the lack of effect of exogenous dopamine agonists (Hagan and Brooks 1996). It seems clear that dopamine inhibits α-MSH secretion by NIL melanotrophs through anatomically identified direct innervation (Bjorkland, Moore et al. 1973). Dopaminergic fibres are also found in the PVN (Buijs, Geffard et al. 1984), but the hypothalamic actions of dopamine may depend on the background activity in other afferent inputs to CRH and AVP neurons.

The modulatory roles of adrenergic and nor-adrenergic neurons on ACTH secretion are similarly confusing and have only been explored in adult animals (Plotsky, Cunningham et al. 1989). Crude extraction studies find no change in the hypothalamic content of catecholamines from 130 days gestation onwards in the fetal sheep (Richards, Gluckman et al. 1987).

1.10 Summary

In all species that have been investigated there is an increase in the concentration of corticosteroid in the fetal circulation shortly before parturition. This is a consequence of increasing activity in the fetal HPA axis since the placenta is an effective physical and metabolic barrier to the passage of maternal corticosteroid. Increasing corticosteroid concentrations are an endocrine 'switch" preparing the fetus for birth. In the larger domestic species, increasing fetal HPA activity also contributes directly to the initiation of parturition by changing the ratio of estrogen/progesterone present in the circulation and presumably also the steroid ratio at a local tissue level.

The development of methods to chronically cannulate the ovine fetus, and later other species, has allowed some insight into the developmental mechanisms involved. An increase in fetal plasma ACTH concentrations are seen at the same time as cortisol concentrations increase. Accompanying increased plasma ACTH and cortisol concentrations, the amount of POMC message in the anterior pituitary is seen to increase from mid to late gestation, as does the amount of CRH and AVP message in the hypothalamic PVN. During the pre-parturient surge in HPA axis activity there may be small decreases in CRH and AVP expression in the PVN as a consequence of negative feedback by corticosteroids, but the expression of POMC in the anterior pituitary seems to escape such suppression. At the same time adrenal sensitivity to ACTH is increased, leading to increased secretion of adrenal corticosteroids and androgenic precursors of estrogen synthesis.

The sustained increase in plasma concentrations of ACTH in the face of elevated levels of corticosteroids in late gestation presents a paradox, since normally corticosteroid negative feedback would be expected to limit the activity of the hypothalamo-pituitary-adrenal axis. The paradox can be explained in terms of "increasing hypothalamic drive" or of "reduced glucocorticoid feedback". Both processes can be seen as part of a programmed pattern of maturation of the fetal HPA. Little is known about the central pathways regulating the fetal HPA axis. Limited evidence suggests that there is a gestation related increase in the responsiveness of the HPA axis to NMDA receptor agonists. The importance of endogenous excitatory amino acid neurotransmitters in the regulation of ACTH secretion is, however, unknown. Other studies have identified an increase in message expression for NPY, one of the neurotransmitters mediating metabolic regulation of ACTH secretion, again consistent with increasing hypothalamic drive. Studies on pituitary glucocorticoid receptors and negative feedback have been confusing, with the number of binding sites decreasing with gestation, whilst message is unchanging or even increasing. Reductions in corticosteroid negative feedback may also occur through modulation of 11βHSD activity, or through interactions with other transcription factors.

The fetal HPA axis can be influenced by placental hormones and by metabolic cues, however, it is not clear what role, if any, these factors play in determining the fine timing of birth. In the primate fetus, it has been suggested that estrogen induces placental 11βHSD activity, which inactivates cortisol in the fetal circulation, thereby overcoming negative feedback and allowing continued ACTH secretion from the pituitary. Other placental hormones may also participate in the drive to parturition: in response to maternal starvation or hypoxia the placenta secretes hormones capable of stimulating the fetal HPA axis.

Teleologically it could be argued that the fetus continues to grow in utero to the point where the placenta (and maternal metabolism) are struggling to provide adequate substrate for continuing growth. If at this point the central pathways regulating ACTH secretion are sufficiently mature then fetal HPA activity increases and parturition is initiated. There is an "intrinsic clock" controlling the timing of increasing adrenal activity in the sense that maturation of the HPA follows a preordained sequence of events that are probably genetically determined, allowing it to respond to peripheral hormonal and metabolic cues.

1.11 Experimental aims

The work presented in this thesis is concerned with the neural pathways regulating ACTH secretion in the fetus. In the studies that follow the role of endogenous excitatory amino acids acting at the NMDA receptor in regulating basal and stimulated ACTH and cortisol secretion in the late gestation ovine fetus is examined using a specific competitive antagonist to the NMDA receptor. The potential action of estrogen to mediate the late gestation increase in fetal HPA axis activity, and especially the increase in responsiveness to NMDA agonists, is also examined. An in vivo microdialysis model has been developed to assess the release of AVP and CRH at the median eminence in an attempt to define how NMDA receptor coupled pathways regulate ACTH secretion. Finally, the potential for metabolic feedback to influence fetal HPA axis activity in late gestation is investigated

by examining the ability of centrally administered NPY to stimulate ACTH secretion.

Four specific hypotheses are tested in each of the experimental chapters:

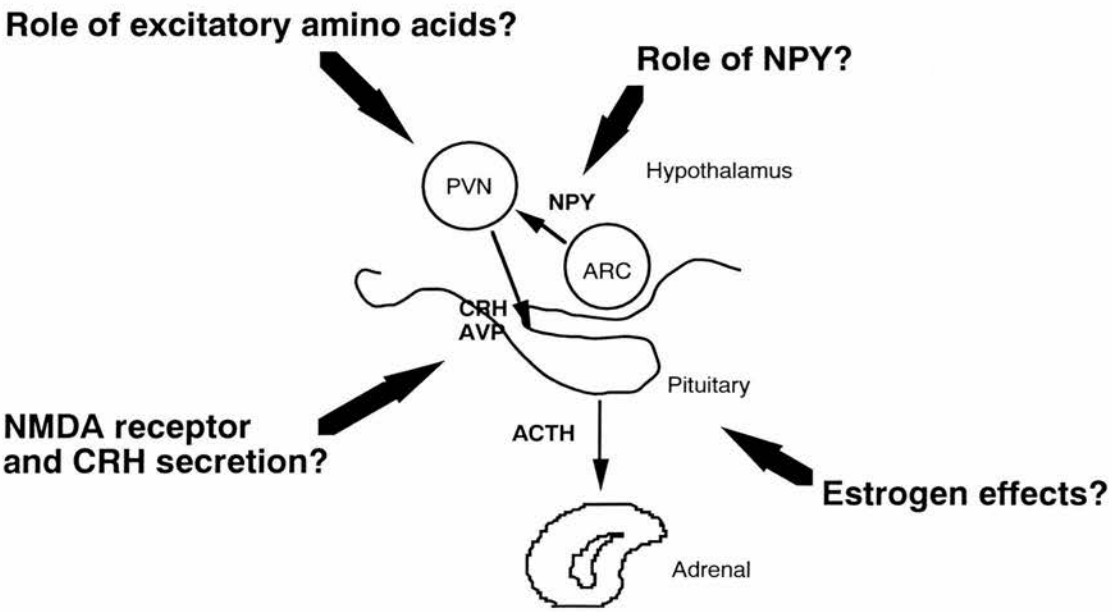
1 Endogenous excitatory amino acid neurotransmitters acting at the NMDA receptor are important regulators of basal and stimulated ACTH and cortisol secretion in the ovine fetus.

2 Endogenous excitatory amino acids acting at the NMDA receptor stimulate ACTH secretion through CRH neurons.

3 The gestation dependent increase in ACTH response to NMDA is due to an effect of estradiol to increase HPA axis sensitivity to NMDA.

4 Central NPY pathways stimulate ACTH secretion.

The diagram below sets out schematically the four areas that are investigated in the experimental chapters.



2

Materials and methods

2.1 Chronically cannulated fetal sheep preparation

2.1.1 Ethical considerations

Necessarily, invasive experiments on the fetus require an animal model and the sheep is a well characterised large animal species suitable for these studies. All experiments were approved by the local Home Office inspectorate and detailed on appropriate project and personal licences. Attempts were made where at all possible to minimize the number of animals utilised and to reduce potential suffering.

2.1.2 Animal husbandry

All animal experiments were conducted at the University of Edinburgh Marshall Buildings, near Roslin, Midlothian, Scotland between October 1992 and August 1995. Scottish blackface and Scottish greyface sheep used in these experiments were housed in a ventilated barn and allowed ad libetum access to hay and water. Dietary intake was supplemented with concentrate twice daily.

2.1.3 Timed matings

Animals with known single insemination dates were used in all experiments. Estrous cycles were synchronised with 60 mg vaginal

medroxyprogesterone sponges (Upjohn Ltd., Crawley,UK) for 13 days. Forty eight hours later ewes were placed with the ram. This was deemed to be day 1 of pregnancy. During seasonal anestrous, ewes additionally received 300 IU pregnant mares serum gonadotropin (Intervet UK, Cambridge,UK) at the time of sponge withdrawal.

2.1.4 Anaesthesia

Anaesthesia was induced with alphaxolone (0.9%) plus alphadolone (0.3%) intravenously at a dose of 0.5 ml/kg (Glaxovet, Uxbridge, UK) and maintained with 3-4% halothane (ICI Pharmaceuticals, Macclesfield, UK) in a nitrous oxide/oxygen mix.

2.1.5 Intravenous cannulation

Under aseptic conditions the uterus was exposed and the fetus partially delivered through a small incision. Care was taken not to disrupt the fetal membranes, except where incised to allow access to the fetus, nor to spill amniotic fluid. A cut down was performed to expose the fetal carotid artery and jugular vein on one side of the neck and polyvinyl catheters (Bolab Inc, Lake Havasu City, USA) were advanced approximately 10 cm so that their tips lay in the aorta and superior vena cava respectively. The skin incision was closed and the catheters further anchored to the skin of the neck. An additional catheter was attached to the neck of the fetus and left free floating in the amniotic fluid. Catheters were exteriorised through a small incision on the maternal flank. Any additional procedures detailed below were than carried out on the fetus. Afterwards the uterus was closed in a single layer incorporating fetal membranes and myometrium, and then a second suture was used to bury the incision.

2.1.6 Lateral ventricle cannulation

For placement of lateral ventricle cannulae and microdialysis probes a stereotaxic approach was adopted. A purpose build frame securing the head and allowing use of a micromanipulator was commissioned from the Department of Veterinary Medicine Workshop, University of Edinburgh. The frame itself could be anchored to the operating table. The fetal head was steadied in the frame by means of two "ear-bars" inserted into a small fossa just inferior and posterior to the external auditory meatus, and by two bars placed over the infra-orbital ridges which served to clamp the skeleton of the face down onto a bar placed under the hard palate. All fitments were adjustable to accommodate fetuses of differing sizes. The accompanying series of line drawings (figures 2.1, 2.2 and 2.3 show details of the stereotaxic frame and tracings from a series of X-rays taken during the placement of a microdialysis probe into the median eminence).

With the head fixed so that external auditory meatus and infra-orbital ridge were on the same plane, a midline incision was made over the scalp to expose the sagittal suture and bregma, the point at which the two parietal and frontal bones meet. For placement of lateral ventricular cannulae, a burr hole approximately 1 mm in diameter was trephined 2 mm anterior and lateral to bregma. A 15 mm long guide cannula constructed from an 18 g syringe needle (Beckton-Dickinson, Wembley, UK) was lowered vertically into place and cemented by means of dental cement ("Formatray", Kerr Europe, Basel, Switzerland) to four anchoring screws which had been sunk into the skull on either side of the midline. The lateral ventricle cannula was made from a 30 mm length of 21 g tubing (Coopers Needle Works, Birmingham,UK.). This was lowered with the aid of a micromanipulator until gravity flow of saline into the lateral ventricle was achieved. The lateral ventricle cannula was then bent so that the length protruding from the top of the guide cannula came to lie flush with the skull, and attached to a 150 cm length of polyethylene tubing (PE20, Clay Adams, NJ, USA) exteriorised through the maternal flank. The whole was then anchored with dental cement and the catheter secured to the skin over the nape of the fetal neck.

2.1.7 Median eminence dialysis

Where a microdialysis probe was to be inserted the lateral ventricle was first cannulated and used as a route for the injection of radio-opaque contrast ("ultravist 300"; Schering Health Care, Burges Hill, UK) into the ventricular system. A lateral skull X-ray allowed for an accurate approach to the median eminence. The radiological landmarks used were based on adult ventriculography (Lignereux, Regodon et al. 1991). A second burr hole to expose the dura was made in the midline in the same vertical plane as the median eminence. A 25 mm guide tube constructed from a 15 g syringe needle (Beckton-Dickinson, Wembley, UK) was then lowered and cemented in place. The guide cannula was supported on a concentric length of 19 g tubing, the sharpened end of which protruded just beyond the tip of the guide and which served to pierce the dura. Once the guide was cemented in position the supporting tubing was removed and the microdialysis probe lowered down the guide by hand until it was felt to come to rest with its tip on the dura overlying the pituitary fossa. The probe tip was approximately 45 mm from the top end of the guide cannula. Once in position the dialysis probe was anchored by dental cement which also served to protect the delicate exposed portion of the probe at the point were it was attached to a catheter exteriorised through the maternal flank.

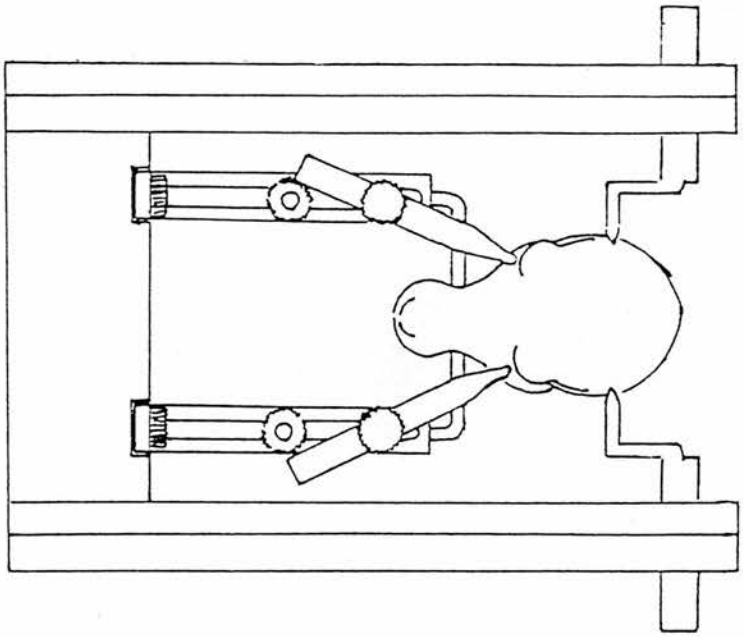
Overleaf

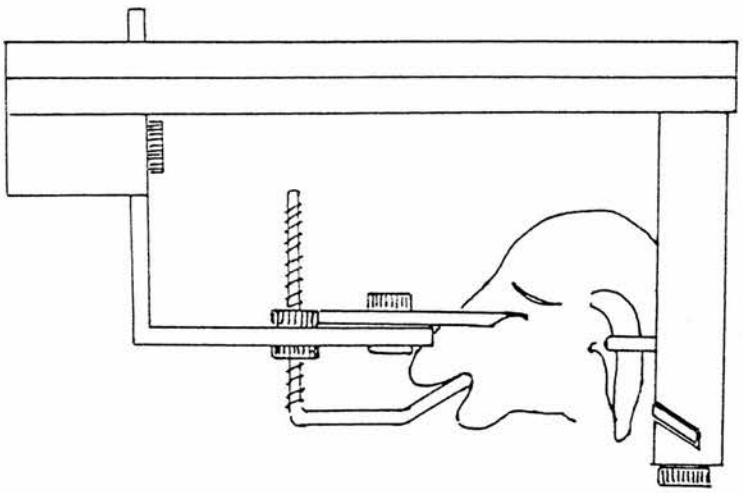
Figure 2.1

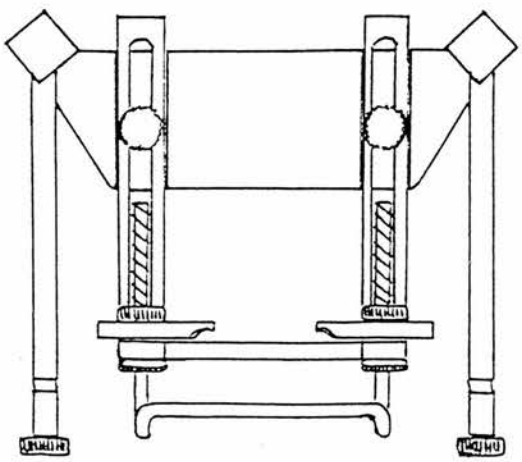
Superior, lateral and posterior projections of the stereotaxic frame. Top panel shows the view from above looking down on the fetal head. Two ear bars and two eye bars fix the skull and prevent lateral movement. A mouthbar under the hard palate serves to prevent rotation of the skull around the axis provided by the earbars. The middle panel shows the frame from the side. The ear, eye and mouth bars are so arranged to allow an uninterrupted view of the base of the skull and pituitary fossa on a lateral skull X-ray.

To place the fetal head in the frame both ear bars are first positioned on the skull and then slipped into the retaining slot on the frame. The ear bars are secured in the frame by a screw. Next the eye bars are positioned resting on the zygomatic arch and finally the mouth bar bought up under the hard palate. Both the eye bar and mouth bar can be moved on their supporting platform, and the whole platform can be moved up and down on the base plate. The eye and mouth bars and supporting frame are locked in position by retaining nuts. The bottom panel shows the frame from the posterior aspect (looking in the same direction as the fetus when the head is in the frame). For clarity the ear bars are shown separate from the frame. In cross section the two main arms of the frame are square and rotated at 45 degrees to the vertical plane. The micromanipulator (not shown) is attached to one or other of these arms to allow guide cannulae and probes to be moved parallel and perpendicular to the interaural axis provided by the ear bars.

2.1





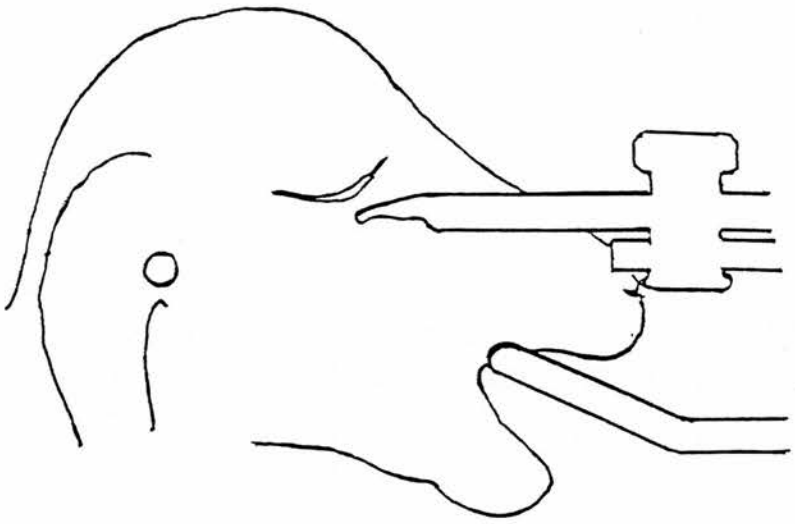


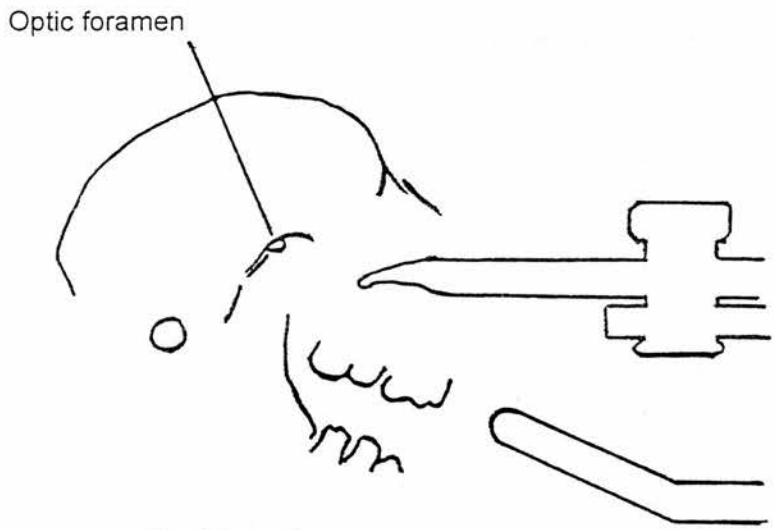
Overleaf

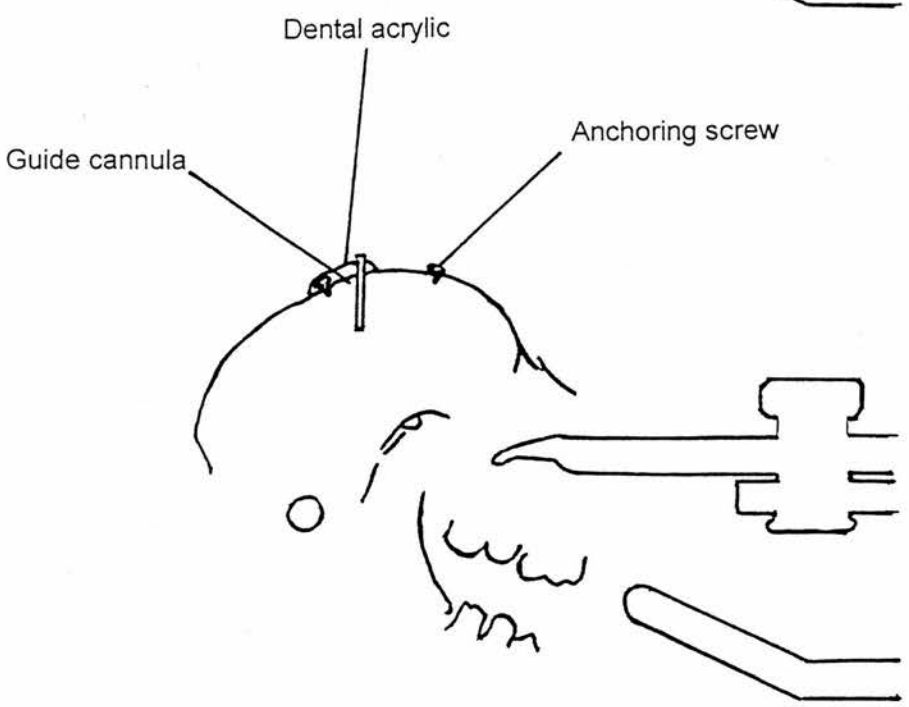
Figure 2.2 and 2.3

Series of line drawings adapted from lateral skull X-rays taken during placement of a microdialysis probe into the median eminence. The upper panel in figure 2.2 shows the fetal head with ear, eye and mouth bars in position. The middle panel shows a lateral skull X-ray of the same fetus. Most of the bones in the fetal skull have not yet fully calcified and only limited detail can be made out. It is possible to identify the optic foramen (allowing the optic nerve to pass). The pituitary fossa is, however, poorly defined on lateral skull X-ray at this gestation. In the lower panel a metal guide cannula, directed at one lateral ventricle, has been implanted and secured to the skull with anchoring screws and dental cement. The top panel in figure 2.3 shows the same animal after a second cannula has been lowered down the guide into the lateral ventricle and contrast material injected. The lateral ventricle and third ventricle are clearly delineated by this procedure, and it is usually possible (with the appropriate amount of contrast) to identify the supra- and infra-optic recess, as well as the infundibular recess of the third ventricle. The infundibular recess is that part of the third ventricle which occupies the median eminence. In the middle panel a second guide cannula aimed at the median eminence has been implanted and secured with dental cement. In the lower panel a microdialysis probe has been lowered down the guide cannula so that the tip of the microdialysis probe lies in the median eminence as identified by radioopaque contrast in the infundibular recess.

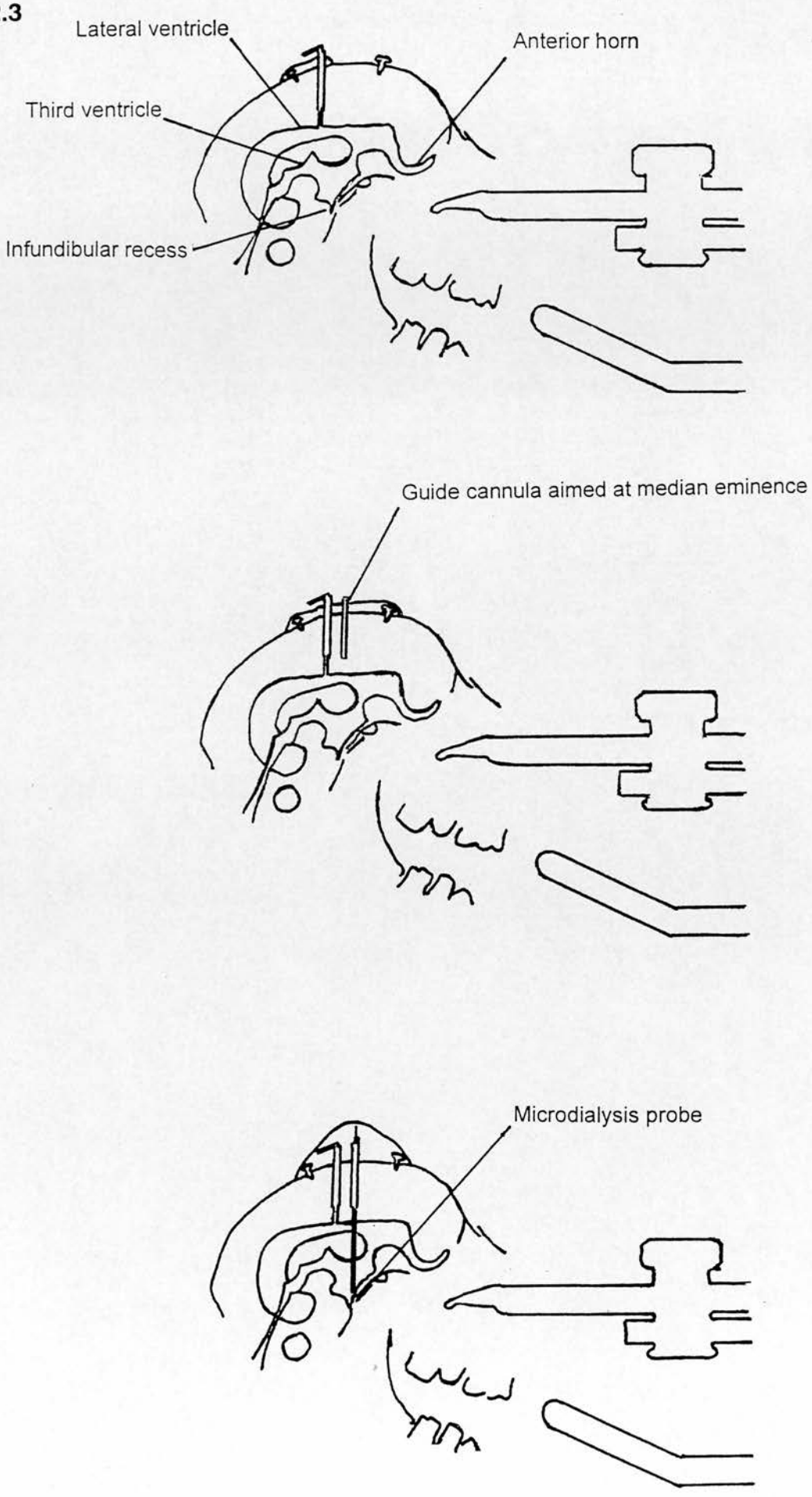
2.2




Optic foramen


Dental acrylic
Guide cannula
Anchoring screw

2.3


Lateral ventricle
Anterior horn
Third ventricle
Infundibular recess
Guide cannula aimed at median eminence
Microdialysis probe

2.1.8 Post-operative care

After surgery the vascular and amniotic catheters were filled with sterile saline containing 20 IU/ml of unfractionated heparin and flushed daily with the same solution (Heparin: Leo Laboratories, Aylesbury, UK, Saline: Travenol Laboratories, Thetford, UK). Catheters were kept in a clean plastic bag tied to the wool over the maternal back and further held in place by a roll of surgical dressing pulled over the maternal abdomen ("netelast", Seton Healthcare, Oldham, UK). The fetus received a once daily injection of 10^6 units benzyl penicillin (Glaxovet, Uxbridge, UK) intravenously and intra-amniotically for three days beginning on the day of surgery. The mother received daily intramuscular injections of streptopen (Pitman Moore, Harefield, UK) for two days. Post-operative analgesia with intramuscular Finadyne (Schering-Plough Animal Health, Mildenhall, UK) was administered upon recovery and then as necessary, usually for the first postoperative day. Sheep were placed in metabolism crates following recovery. These allowed forward and backward movement but not the freedom to turn around. All experiments were conducted at least 5 days after surgery.

2.1.9 Blood sampling and intravenous drug administration

Fetal blood samples were withdrawn via a three-way tap (Viggo Products, Helsingborg, Sweden) connected to the catheter, allowing for the dead space (approximately 0.8 ml) in the tubing, and collected into heparinised polystyrene tubes on ice (Sarstedt, Leicester, UK). Blood volume was replaced with an equal volume of sterile heparinised saline (20units/ml). Samples were centrifuged (2500 rpm, 15 min, 4C) within 20 min of collection and the plasma frozen until assay in 2 ml sample tubes (Teklab Medical Laboratories, Durham, UK). Samples spiked with 200 pg/ml ACTH at the time of collection did not lose appreciable ACTH immunoactivity when stored on ice for upto 2 h before centrifugation.

Drugs for intravenous administration were introduced through the three-way tap attached to the catheter and flushed through the dead space in the cannulae with heparinised saline.

2.2 Hormone assays

The concentrations of ACTH, FSH, PRL, GH, and cortisol in unextracted plasma samples were determined by radioimmunoassay (RIA) or by immunoradiometric assay (IRMA) as described below. Median eminence dialysate samples were assayed for AVP and CRH by RIA and these are also described. For convenience, the sources of standard reagents are given in appendix 1. All assays were conducted in polystyrene tubes (Sarstedt, Leicester, UK). Two standard curves in triplicate, prepared by serial dilution of stock peptide, and appropriate quality controls, were included in each assay. The standard curve included an estimate of total binding of radiolabel to primary antibody (Bo) and non-specific binding of radiolabel to the polystyrene tube (NSB). The upper and lower limits of sensitivity of the assay are calculated as the hormone concentration displacing 90% and 10% respectively of radiolabel from the primary antibody. Assay precision (intra- and inter-assay coefficients of variation) is calculated from the quality control samples included in each assay and is given as the standard deviation in the measurement of a particular quality control expressed as a percentage of the mean (Chard 1978). Since all of these assays have been previously validated, cross-reactivities are quoted as available in the literature.

2.2.1 Adrenocorticotrophin

The concentrations of ACTH in fetal plasma samples were measured with a specific 2-site immunoradiometric assay according to a previously described protocol (Brooks, Howe et al. 1994; Brooks and Howe 1996). Intact ACTH $_{(1\text{-}39)}$ is detected by virtue of a rabbit polyclonal capture antibody

directed at one end of the molecule, and a ^{125}I labelled guinea pig detection antibody targeted to the other end of the molecule.

Assay buffer: 0.1M phosphate buffer containing 0.5% (w/v) bovine serum albumin, 0.001% (v/v) Triton X-100 and 0.0001% (w/v) Sodium azide.

Antibody: The antibodies are commercially available (Euro-path Ltd., Bude, UK) and are supplied as a lyophilised powder for reconstitution in distilled water.

Standards/Quality controls: Synthetic ACTH $_{(1-39)}$ (Cambridge Research Biochemicals, Northwich, UK) was dissolved in 0.05M phoshate buffer containing 0.5% (w/v) bovine serum albumin and 0.0001% (w/v) sodium azide to a concentration of 100 ng/ml. This stock was stored frozen until use when serial dilutions from 5000 to 5 pg/ml were prepared in assay buffer. Quality controls were made by spiking dexamethasone-suppressed plasma with synthetic ACTH to final concentrations of 25, 100, and 500 pg/ml.

Assay procedure: Samples were assayed in duplicate 100 μl aliquots. To ensure that the same ratio of plasma to assay buffer was present in standards and samples, 100 μl of dexamethasone-suppressed plasma was added to each standard, and 100 μl of assay buffer to each sample or quality control. A100 μl aliquot of mixed capture and detection antibody was then added to all tubes which were incubated overnight at 4C.

Separation: The capture antibody was precipitated by magnetic separation after further overnight incubation at 4C with 200 μl polyclonal donkey anti-rabbit immunoglobulin. The magnetically tagged polyclonal donkey anti-rabbit immunoglobulin was prepared in house by Dr Rodney Kelly and used at 1:2 dilution in 0.05M phosphate buffer with 0.01% (v/v) Triton-X. After overnight incubation the tubes were clipped to a magnetic separation rack (Amerlex-M assay rack, Amersham, Little chalfont, UK) and allowed to stand for 15 min

before the supernatant was poured off. The tubes were then removed from the magnet and washed with 300 μl "wash buffer" (0.075M phosphate buffered saline containing 0.15% (w/v) sodium azide and 0.015% 9v/v) Triton-X 100) at which point they were once more clipped to the magnet, left for 15 min and finally inverted and left to dry.

Sensitivity and cross reactivity: The assay performed with a lower limit of detection of 11pg/ml. The assay does not detect ACTH $_{(18-39)}$ or ACTH $_{(1-24)}$, α-MSH or non-amidated α-MSH (White, Smith et al. 1987).

Precision: The intra-assay coefficients of variation were 12% at 25 pg/ml, 10% at 100 pg/ml and 6% at 500 pg/ml. The inter-assay coefficients of variation were 14%, 11% and 10% respectively.

2.2.2 Cortisol

The concentration of cortisol in plasma samples was determined by radioimmunoassay after extraction with diethyl ether as previously described (Brooks and White 1990).

Assay buffer: 0.01M phosphate buffer containing 0.1% (w/v) gelatin, 0.9% (w/v) sodium chloride, and 0.01% (w/v) thiomersal at pH 7.4

Antibody: Sheep polyclonal anti-cortisol (Code S004-201) obtained from Scottish antibody production Unit (SAPU), Carluke, Scotland.

Radiolabel: Cortisol-3-(O-carboxymethyl)oximino-(2-(^{125}I)iodohistamine) was purchased from Amersham, Little Chalfont, UK.

Cortisol extraction: Aliquots of 50 μl of sample were agitated with 2.5 ml diethyl ether in borosilicate tubes for 15 min and then placed in an ethanol/dry ice bath to freeze the aqueous phase. The supernatant diethyl ether was

collected into a fresh borosilicate tube and evaporated to dryness under nitrogen. Extraction efficiency was calculated from the recovery of radioactivity in a sample of dexamethasone-suppressed plasma spiked with tritiated cortisol (Amersham, Little Chalfont, UK). Samples for radioimmunoassay were stored frozen until use and then resuspended in 500 μl assay buffer, vortexed thoroughly and left for at least 30 min at room temperature.

Standards/Quality controls: Standards ranging between 0.15 and 80 ng/ml in assay buffer were prepared and stored at 4C. These standards were used for all subsequent assays. Quality controls were made by spiking dexamethasone suppressed plasma with 0, 3.9 and 19 ng/ml cortisol and were stored frozen in aliquots until use.

Assay procedure: Samples and quality controls were extracted in parallel and stored frozen at -20C. The cortisol concentration was determined in duplicate 100 μl aliquots following resuspension in 500 μl assay buffer. Aliquots of standard, quality control and sample were incubated overnight at 4C with 100 μl of S004-201 anti-cortisol antibody (at 1:8000 dilution) and 100 μl of radiolabelled tracer (at 15,000 cpm/100 μl), both diluted in assay buffer. *Separation:* The primary antibody was precipitated by overnight incubation at 4C with polyclonal donkey anti-goat second antibody (SAPU, Carluke, Scotland) at a dilution of 1:1200 in 200 μl assay buffer containing 1.6% normal sheep serum. The donkey anti-goat antibody and normal sheep serum were combined shortly before addition to avoid precipitation of immunoglobulin present in the sheep serum by the second antibody. The following day 1 ml of 0.9% saline with 0.2% (v/v) Triton X-100 was added to all samples, standards and quality controls. The tubes were centrifuged at 3000 rpm for 30 min and then inverted and left to dry.

Sensitivity and cross reactivity: The assay performed with a lower limit of 0.27 ng/ml. Cross reactivity for various steroids is reported as: corticosterone

0.18%, cortisone 0.07%, 21-deoxycortisone 0.3%, 11-deoxycorticosterone 0.03%, 11-deoxycortisol 0.58%, 17 alpha-hydroxyprogesterone 2.1% (Abraham 1969).

Precision: The intra-assay coefficients of variation were 5% at 0 ng/ml, 7% at 3.9 ng/ml, and 9% at 19 ng/ml. The inter-assay coefficients of variation were all less than 10%.

2.2.3 Growth Hormone

The concentration of growth hormone (GH) in plasma samples was determined by radioimmunoassay after a dilution step to bring the concentrations to lie within the working range of the assay. The assay is a modification of a previously described procedure miniaturised for fetal samples (Thomas, Mercer et al. 1990).

Assay buffer: 0.05M phosphate buffer containing 0.5% (w/v) bovine serum albumin, 0.8% (w/v) sodium chloride, and 0.01% (w/v) thiomersal at pH 7.4.

Antibody: Polyclonal rabbit-anti-oGH-2 (AFP-C0123080) obtained from the National Institute of Diabetes, Digestive and Kidney Diseases, USA.
Radiolabel: Ovine GH $_{(1-4)}$ (Ref AFP-8758C) obtained from the National Institute of Diabetes, Digestive and Kidney Diseases, USA was iodinated by the lactoperoxidase method as follows. A reaction mixture of 5 μg of oGH $_{(1-4)}$ dissolved in 50 μl of 0.1M sodium hydrogen carbonate, 10 μl of Sodium iodide (^{125}I) (1 mCi/ml; Amersham, Little Chalfont, UK), 10 μl dilute hydrogen peroxide (1: 15000 in water) and 10 μl of lactoperoxidase (Sigma, Poole, UK) was incubated for 20 seconds at room temperature. The reaction was terminated by the addition of 1 ml of 0.1M phoshate buffered saline containing 0.1% (w/v) bovine serum albumin which provides a large number of sites for non-specific iodination. Labelled GH $_{(1-4)}$ was separated from free radioactive iodine on a 45 cm Sephadex G100 column eluted with 1% bovine serum

albumin in 0.1M phosphate buffered saline. The column was prepared by swelling approximately 4g Sephadex G100 beads (Pharmacia, Uppsala, Sweden) in 100 ml 0.05M phosphate buffer. The G100 was then poured into a 50 cm by 1 cm glass column (Amicon, Stonehouse, UK) until the packed bed line was at 45 cm, at which point the column was covered with approximately 50 ml of 0.05M phosphate buffer and clamped off until use. The fractions exhibiting greatest binding to the primary were pooled, divided into aliquots and stored frozen at -20C until use.

Standards/Quality controls: Ovine GH $_{(1-4)}$ from the National Institute of Diabetes, Digestive and Kidney Diseases, USA was dissolved in assay buffer to a final concentration of 100 μg/ml and stored frozen in aliquots. Standards ranging between 125 and 0.49 ng/ml of GH $_{(1-4)}$ were prepared by double dilution in assay buffer. Quality controls of 0.49, 7.8 and 125 ng/ml of GH $_{(1-4)}$ in assay buffer were stored frozen in aliquots until use.

Assay procedure: Plasma samples were diluted 1:10 with assay buffer to lie within the working range of the assay. Thus 20 ml fetal plasma was diluted with 180 μl assay buffer, vortexed and from this two 50 μl aliquots assayed in duplicate. Samples, standards and quality controls were incubated overnight at 4C with antibody at a dilution of 1:30,000 in 100 μl assay buffer. The following day 50 μl radiolabelled GH $_{(1-4)}$ (diluted to roughly 15,000 cpm/100 μl) in assay buffer was added and a further overnight incubation at 4C allowed.

Separation: The primary antibody was incubated for 24 h at 4C with 100 μl polyclonal donkey anti-rabbit immunoglobulin at a dilution of 1:32 (SAPU, Carluke, Scotland) and 100 μl normal rabbit serum at a dilution of 1:400 (SAPU, Carluke, Scotland) in assay buffer. The next day, following the addition of 1 ml of 0.9% saline with 4% (w/v) polyethylene glycol and 0.2%

(v/v) Triton X100, the samples were centrifuged at 3000 rpm for 30 min and then inverted and left to dry.

Sensitivity: The lower limit of detection was 1.9 ng/ml.

Precision:: The intra-assay coefficients of variation were 12% at 0.49 ng/ml, 7% at 7.8 ng/ml, and 12% at 125 ng/ml. The inter-assay coefficients of variation were 14%, 10% and 15% respectively.

2.2.4 Follicle stimulating hormone

The concentration of follicle stimulating hormone (FSH) in plasma samples was determined using a modification of an established assay for ovine FSH (McNeilly, McNeilly et al. 1976; McNeilly, Jonassen et al. 1986).

Assay buffer: 0.075M phosphate buffer with 1% (w/v) bovine serum albumin , 0.875% (w/v) sodium chloride and 0.01% (w/v) thiomersal at pH7.4.

Antibody: polyclonal rabbit anti ovine FSH. (NIDDK-NIH anti-oFSH-1, Ref AFP-C5288113) obtained from NIH.

Radiolabel: Radiolabelled purified ovine FSH (NIAMMD-oFSH-19) obtained from NIH was prepared by the lactoperoxidase method. The technique is identical to that used to prepare radiolabelled GH tracer except that separation was achieved on a 20 cm Sephadex G100 column eluted with 0.1M phosphate buffered saline containing 1% (w/v) bovine serum albumin. The fractions exhibiting the strongest binding to primary antiserum were pooled and stored at -20C until use.

Standards/Quality controls: Standards ranging between 0.1 and 25ng/ml in assay buffer were prepared by dilution of stock NIDDK-oFSH-RP-2 (Ref AFP-4117A from NIDDK). Standards were aliquoted and stored frozen until use.

Quality controls were made by spiking buffer with 0.4, 0.84, and 2.64 ng/ml oFSH-RP-2.

Procedure: Duplicate 150 μl aliquots of sample were diluted to a final volume of 300 μl with assay buffer and incubated overnight at 4C with 50 μl of primary antibody diluted 1:12000 in assay buffer. The following day 50 μl radiolabelled FSH tracer (roughly 15000 cpm/50 μl) was added to all tubes and a further 24 h incubation allowed.

Separation: The primary antibody was precipitated with a donkey anti-rabbit second antibody exactly as described for the separation of GH radiolabel.

Sensitivity and cross reactivity: The lower limit of detection was 0.29 ng/ml and reported cross reactivities are less than 0.1% for ovine LH, thyroid stimulating hormone (TSH), GH and prolactin (PRL) (McNeilly, McNeilly et al. 1976).

Precision: The mean intra-assay coefficient of variation was 4.8% (single assay).

2.2.5 Prolactin

The concentration of prolactin (PRL) in samples was determined with an established radioimmunoassay for ovine PRL (McNeilly and Andrews 1974).

Assay buffer: 0.075M phosphate buffer with 1% (w/v) bovine serum albumin, 0.875% (w/v) sodium chloride, and 0.01% (w/v) thiomersal at pH7.4.

Antibody: The primary antibody utilised was a polyclonal rabbit anti PRL immunoglobulin gifted by Professor A. McNeilly, MRC Reproductive Biology, Edinburgh, UK.

Radiolabel: Purified ovine PRL (oPRL-17 from NIAMDD) was iodinated by the lactoperoxidase method as described for the preparation of GH tracer. Labelled oPRL-17 was separated on a 45 cm Sepadex G100 column eluted with 1% bovine serum albumin in 0.1M phosphate buffered saline. Aliquots of radiolabelled tracer were stored at -20C until use.

Standards/Quality controls: Standards ranging between 0.4 and 200 ng/ml of NIH PRL-S15 were prepared by double dilution in assay buffer and stored at -20C until use. Quality controls of 3.2, 25 and 100 ng/ml of NIH PRL-S15 were prepared in assay buffer and stored frozen in aliquots until use.

Procedure: Plasma samples were assayed in 30 μl duplicate aliquots diluted with 100 μl assay buffer. After the addition of 100 μl primary antibody (1:128,000 in assay buffer) and 100 μl radiolabelled PRL-17 (at roughly 15,000 cpm/100 μl in assay buffer) to each tube an overnight incubation at 4C carried out.

Separation: The primary antibody was precipitated with a donkey anti-rabbit second antibody exactly as described for the separation of GH radiolabel.
Sensitivity and cross reactivity: The lower limit of sensitivity was 0.54 ng/ml. The antibody does not detect ovine GH, LH, FSH, TSH or ACTH (McNeilly and Andrews 1974).

Precision: The intra-assay coefficients of variation were 14% at 3.2 ng/ml, 11% at 25 ng/ml, and 12% at 100 ng/ml. The inter-assay coefficients of variation were 14%, 14% and 15% respectively.

2.2.6 Vasopressin

The concentration of AVP in median eminence dialysate was measured using a previously described radioimmunoassay (Currie, Gillies et al. 1994).

Assay buffer: 0.05M phosphate buffer containing 0.1% (w/v) bovine serum albumin, 0.58% (w/v) sodium chloride, 0.0372% (w/v) EDTA and 0.01% (w/v) thiomersal at pH7.4.

Antibody: Polyclonal rabbit anti-AVP (NK-2) gifted by Dr N Kasting, Department of Physiology, University of British Columbia, Vancouver, Canada.

Radiolabel: ^{125}I-Arg^{8}-AVP was prepared by the chloramine-T method as follows: A reaction mixture of 5 μg of Arg^{8}-AVP (Cambridge Research Biochemicals, Northwich, UK) dissolved in 10 μl of 0.1M acetic acid, 15 μl of 0.5M phosphate buffer, 10μl of sodium iodide (^{125}I) (1 mCi/ml; Amersham, Little Chalfont, UK) and 10 μl of 0.5% (w/v) chloramine-T in 0.05M phosphate buffer was incubated for 10 seconds at room temperature. The reaction was terminated by the addition of 600 μl of 0.5% (w/v) bovine serum albumin (fraction V, RIA grade) in 0.05M phoshate buffer. Labelled AVP was separated from free radioactive iodine on a "Sep-pak" column (Millipore, Herts, UK) prewetted sequentially with 2 ml 1% (v/v) trifluoroacetic acid in water (1%TFA), 5ml of 80% methanol/20% of 1%TFA, 3 ml of 1%TFA and finally, 1 ml of 0.01% (w/v) polypep in 1%TFA. Free iodine was eluted with 700 μl of 1%TFA. Iodinated AVP was then eluted from the column by a stepwise increase in methanol/1%TFA eluant. The fraction exhibiting the greatest binding to AVP antiserum was usually 44% methanol which was stored at -20C until use.

Standards/Quality controls: Arg^8-AVP was dissolved in 0.1M acetic acid to a stock concentration of 1mg/ml and aliquots stored at-20C until use. Fresh standards ranging between 500 to 0.5 pg/ml were prepared by serial dilution in assay buffer for each assay. Quality controls of 2, 10, and 50 pg/ml in assay buffer were prepared and stored frozen until use.

Procedure: Samples were assayed in singlicate 100 μl aliquots (though in some cases samples required to be diluted to lie within the range of the assay). Aliquots of standard, quality control and sample were diluted with 200 μl assay buffer and incubated overnight at 4C with 100 μl of antibody at a dilution of 1: 50,000 in assay buffer. The following day 100 μl of radiolabelled tracer at a concentration of 15000 cpm/100 μl was added to all tubes and a further 24 h incubation at 4C allowed.

Separation: The primary antibody was precipitated by magnetic separation after incubation with polyclonal donkey anti-rabbit antibody bound to magnetic particles (gift of Dr. R. Kelly, MRC Reproductive Biology Unit). Briefly, 500 μl of antibody diluted 1:80 in 0.05M phosphate buffer was added to each tube and incubated for 30 min at room temperature. Tubes were clipped into a commercially available magnetic separation rack (Amerlex-M assay rack, Amersham, UK) and the supernatant poured off after allowing the tubes to stand for 15 min. The precipitate was then resuspended in 300 μl wash buffer by gentle agitation after the tubes had been separated from the magnetic rack. The tubes were again clipped to the magnetic rack and after a further 15 min were inverted and left to dry.

Sensitivity and cross reactivity: The antibody to AVP is reported to have <2% cross reactivity with oxytocin and a number of other neuropeptides (Currie, Gillies et al. 1994). The lower limit of sensitivity was 1.75pg/ml.

Precision: The intra-assay coefficients of variation were 15% at 2 pg/ml, 10% at 10 pg/ml, and 12% at 50 pg/ml. The inter-assay coefficients of variation were 18%, 10% and 16% respectively.

2.2.7 Corticotrophin Releasing Hormone

The concentrations of CRH in median eminence dialysate were determined by established radioimmunoassay (Brooks, Power et al. 1989).

Assay buffer: 0.05M phosphate buffer containing 0.5% (w/v) bovine serum albumin, 0.58% (w/v) sodium chloride, 0.95% (w/v) EDTA, 0.1% (v/v) Triton X-100, and 0.01% (w/v) thiomersal at pH 7.4.

Antibody: Polyclonal rabbit anti oCRH (CRF-2G) immunoglobulin raised by Dr. A.N. Brooks

Radiolabel: ^{125}I-Tyr-oCRH was prepared by the chloramine-T method described below. A reaction mixture of 4 μg of Tyr-oCRF (Cambridge Research Biochemicals, Northwich, UK) dissolved in 10 μl of 0.1M acetic acid, 10 μl of 0.05M phosphate buffer, 10 μl of sodium (^{125}I) iodide (1mCi/ml; Amersham) and 10 μl of 1% (w/v) chloramine-T in 0.05M phosphate buffer was incubated for 15 seconds at room temperature. The reaction was terminated by the addition of 25 μl of 1% (w/v) sodium metabisulphite in 0.05M phoshate buffer. Labelled Tyr-oCRH was separated on a "Sep-pak" cartridge (Millipore, Herts, UK) prewetted sequentially with 5 ml methanol, 10 ml water, and 5 ml of 0.1% (v/v) TFA in 0.05M phosphate buffer. Free iodine was eluted with 6 ml of 0.1%TFA in 0.05M phosphate buffer, and then radiolabelled TYR-oCRH was eluted with 3 ml of a mixture of 4 parts acetonitrile to 1 part 0.1% TFA in 0.05M phosphate buffer. The pooled radiolabelled TYR-oCRH was checked for binding to the primary antiserum and then stored frozen at -20C until use.

Standards/Quality controls: stock oCRH purchased from Cambridge Research Biochemicals (Northwich, UK) was dissolved to a final concentration of 10 μg/ml in assay buffer and stored frozen until use. Fresh standards between 39 and 5000 pg/ml were prepared by double dilution in assay buffer.

Procedure: Samples were assayed in duplicate 100 μl aliquots diluted with 100 μl assay buffer and incubated overnight at 4C with 100 μl of antibody at 1: 100,000 dilution in assay buffer. The following day 100 μl radiolabelled tyr-CRH (15,000 cpm/100 μl) was added to all tubes and a further 24 h incubation carried out at 4C.

Separation: Primary antibody was precipitated by magnetic separation as described for AVP.

Sensitivity and cross reactivity: The lower limit of detection was 120 pg/ml. The antibody is reported to have <0.001% cross reactivity with ACTH $_{(1\text{-}39)}$, ACTH $_{(1\text{-}24)}$, beta-endorphin, alpha-melanocyte stimulating hormone, met enkephalin, leu-enkephalin, AVP, oxytocin, thyroid releasing hormone, and luteinizing hormone releasing hormone (Brooks, Power et al. 1989).

Precision: The intra-assay coefficient of variation was 16% (single assay).

2.2.8 Determination of hormone concentration

The gamma radiation emitting isotope of iodine (^{125}I) was used for all radioimmunoassays.. The activity remaining in each assay tube after separation of free and bound radiolabelled tracer was counted over 60 seconds (Multigamma 1261, LKB, Turku, Finland) and the data were collected online ("Datagrabber, Mutek,Box, Wiltshire,UK) onto an Apple Macintosh computer. Assay results were computed using the program "AssayZap" (Biosoft, Cambridge, UK) on an apple Macintosh computer.

2.3 Statistical analysis

All statistical computations were performed using the Abacus Concepts Statview package (version 4.1) (Abacus Concepts Inc, Berkeley, California, USA) on an Apple MacIntosh G3 powerbook. The statistical analyses for each experiment are described more fully in each chapter. In some cases serial blood samples have been taken and hormone concentrations analysed with a pulse detection program (Munro, Zaristow Software, Haddington, Scotland). Data is displayed as mean$\pm$standard error of the mean (SEM) and the significance level is set at $p<0.05$.

3

The role of endogenous central excitatory amino acid pathways in the control of adrenocortical activity in the fetal sheep in late gestation

3.1 Abstract

The role of endogenous excitatory amino acids acting at the NMDA receptor in the regulation of ACTH secretion in the late gestation ovine fetus is investigated. Time-mated chronically catheterised fetal sheep at day 130 gestation were challenged with insulin (10 IU iv bolus) or sodium nitroprusside (iv infusion 100 μg/min for 10 min). Pretreatment with CGP 37849 (1 mg/kg, iv bolus), a competitive NMDA antagonist, significantly inhibited insulin hypoglycaemia, but not nitroprusside stimulated ACTH secretion. The effects of CGP 37849 on pulsatile ACTH, cortisol and GH secretion were examined in a separate group of animals at 138 days gestation. Blood samples withdrawn every 10 min were analysed for intact $ACTH_{(1-39)}$, cortisol, and GH. After a 3 h baseline sampling period CGP 37849 given as a loading bolus (1 mg/kg iv), followed by a 3 h infusion (1 mg/kg/h) resulted in a rapid decrease in mean plasma levels of ACTH and cortisol but not GH. This was a consequence of decreased basal secretion, as pulse amplitude and interpulse interval remained unchanged. These results demonstrate that endogenous excitatory amino acid neurotransmitters acting at the NMDA receptor regulate basal ACTH secretion in the late gestation ovine fetus.

3.2 Introduction

Adrenocorticotrophin secretion in the late gestation fetus is highly pulsatile (Brooks and Challis 1991; Apostolakis, Longo et al. 1992) and presumably reflects secretion of the hypophysiotrophic releasing factors AVP and CRH in to portal plasma, just as it does in the adult (Caraty, Griro et al. 1988; Engler, Pham et al. 1989). Several neurotransmitters are thought to be involved in the control of CRH and AVP secretion (Assenmader, Szafarczyx et al. 1987; Palkovitz 1987; Jacobowitz 1988; Plotsky 1991; Antoni 1993; Whitnall 1993). The excitatory amino acids as a class have attracted interest because of their widespread distribution and protean interactions with other neurotransmitter systems (Ruzika and Jhamander 1993). There is neuroanatomical evidence to support a role in neuroendocrine regulation: NMDA receptors have been localised to the hypothalamic PVN and arcuate nuclei (Monyer, Sprengel et al. 1992; Meeker, Greenwood et al. 1994) and these same areas also contain glutamate immunoreactive fibres (Van den Pol, Waurin et al. 1990; Van den Pol 1991; Goldsmith, Thind et al. 1994). In other neuroendocrine situations excitatory amino acid transmitters have a role in controlling pulsatile secretion, notably of LH. Agonist at the NMDA class of excitatory amino acid receptor stimulate the secretion of LH (Estienne, Schillo et al. 1990; Lincoln and Wu 1991; Kumar, Lincoln et al. 1993). Conversely, antagonists inhibit pulsatile LH secretion (Arslan, Pohl et al. 1988; Ping, Mahesh et al. 1994). Other pituitary hormones that are secreted in a predominantly pulsatile manner may also be regulated by NMDA sensitive pathways (Brann and Mahesh 1997). In adult animals NMDA stimulates ACTH (Farah, Rao et al. 1991; Downing, Joss et al. 1996) and growth hormone (GH) secretion (Estienne, Schillo et al. 1989; Acs, Lonart et al. 1990; Downing, Joss et al. 1996), but the effect of antagonising the actions of the endogenous ligand(s) on pulsatile ACTH or GH secretion has not been examined.

Administration of NMDA to the ovine fetus stimulates ACTH secretion, and the response increases with advancing gestational age; At the same time the pituitary sensitivity to AVP and CRH is unchanged or may even

decrease (Brooks and Howe 1996). An appealing possibility is that increasing glutaminergic drive to PVN neurons is the central event preceding parturition. Accordingly, in this study the role of endogenous excitatory amino acids acting at the NMDA receptor in mediating pulsatile ACTH secretion in the late gestation ovine fetus is examined.

3.3 Materials and methods

Animals and surgical preparation

Mixed breed sheep with known single insemination dates were used in these experiments. Between day 120-125 gestation (term approximately 145 days) fetal sheep were prepared with chronic indwelling jugular, carotid and amniotic cannulae as previously described in section 2.1. Where there were multiple fetuses only one fetus in each sheep was cannulated. Within 24 h of surgery animals were individually housed in metabolism crates and maintained for the duration of the experiment under a constant 12:12 h light dark cycle. The cannulae were flushed daily with heparinised saline (5000 IU/ml) and a small arterial sample withdrawn for blood gas analysis (IL1306; Instrumentation Laboratories, Warrington, Cheshire, UK). Only fetuses with maintained healthy acid-base status (measurements in close agreement with normal ranges of: pH 7.352$\pm$0.01; pO_2 20.28$\pm$0.49 mmHg; pCO_2 49.39$\pm$0.59 mmHg) were included in the experiment. Antibiotics were given to the fetus (10^6 units penicillin, Glaxovet, Uxbridge, UK) and mother (Streptopen, Glaxovet, Uxbridge, UK) for 3 days after surgery. All experiments were conducted at least 5 days after surgery.

Blood sampling regime

Samples (1 ml) were withdrawn from the arterial cannula and replaced with heparinised saline. All samples were collected onto ice and centrifuged within 15 min (3000 rpm, 15 min, 4C), separated and stored frozen at -20C until analysis.

Experimental strategy

To examine the role of endogenous excitatory amino acids acting at the NMDA receptor we examined the effect of a specific competitive NMDA antagonist (CGP 37849, Ciba-Geigy, Basel, Switzerland) on stimulated and basal hormone secretion in fetal sheep during late gestation. Insulin-induced hypoglycaemia, sodium nitroprusside and NMDA were used to stimulate hormone secretion on consecutive days from 130-132 gestation at a time when circulating concentrations of ACTH and cortisol are low. Effects of CGP 37849 on basal secretion were studied during the preparturient surge of ACTH and cortisol secretion at around day 138. Some fetuses were used for both experiments.

Experiment 1: Effect of NMDA receptor antagonism on stimulated hormone secretion

On day 130 gestation beginning at 0800 h, fetuses received an intravenous bolus of either CGP 37849 (treatment, n=5) or saline vehicle (control, n=5). CGP 37849 was given at a dose of 1 mg/kg in a solution of 2 mg/ml in saline (estimated fetal weight of 4 kg). Fetuses were then challenged 5 min later with insulin (10 IU iv bolus; Actrapid, Novonordisk, Crawley, UK). Blood samples (1 ml) were withdrawn at -60, -30, -15, 0, 5, 10, 15, 20, 30, 60, 90, and 120 min relative to the time of administration of antagonist. On the following day animals underwent an identical treatment and sampling protocol except that the insulin challenge was replaced with an infusion of sodium nitroprusside (100 μg/0.5ml saline/min iv) for 10 min. On the subsequent day NMDA (4 mg iv bolus; Sigma, Poole, UK) was administered as an intravenous bolus and blood samples collected as for the previous challenge tests.

Experiment 2: Effect of NMDA receptor antagonism on endogenous hormone secretion.

On day 138 gestation beginning at 0800 h blood samples were withdrawn from fetuses at 10 min intervals for 6 h. After a baseline period of 3 h, fetuses received either an intravenous infusion of CGP 37849 (5 treated animals) or saline vehicle (6 control animals). The CGP 37849 was prepared as a solution of 2 mg/ml and given as a loading bolus of 1 mg/kg followed by an infusion at 1 mg/kg/h for a further 3 h treatment period (estimated fetal weight 5 kg). At the end of this time all fetuses received an intra-arterial bolus injection of NMDA (4 mg/kg) whilst the antagonist or vehicle infusion was continued intravenously. Further blood samples were withdrawn at 5, 10, 15, 20, 30, 60, 90, and 120 min after the injection of NMDA. Additional fetal blood samples were withdrawn for blood gas analysis and determination of haematocrit at the start of the baseline period, the treatment period, before the NMDA challenge and at the end of the experiment. Fetuses with significant hypoxia or acidosis or with a drop of more than 10% haematocrit were excluded from analysis.

Hormone determination.

Samples were assayed in duplicate for immunoreactive $ACTH_{(1-39)}$ by a specific 2-site immunoradiometric assay (Brooks and Howe 1996) and described in detail in section 2.2.1. The assay limit of detection was 11.0 pg/ml. Inter- and intra-assay coefficients of variation were 12% and 9% respectively. Cortisol concentrations were determined by radio-immunoassay following extraction with diethyl-ether using established methods (Brooks and White 1990) covered in section 2.2.2. The lower limit of detection was 0.27 ng/ml. Inter- and intra-assay coefficients of variation were less than 10% and 7%, respectively. Samples were assayed in duplicate 50 μl aliquots for ovine GH (oGH) by radioimmunoassay as described in section 2.2.3. The lower limit of detection was 1.9 ng/ml. Inter- and intra-assay coefficients of variation were less than 12% and 10%, respectively.

Statistical analysis

Experiment 1: The effects of CGP 37849 on hormone responses to the insulin and nitroprusside challenge tests were assessed by two way ANOVA for repeated measures with post hoc Scheffe's test using the Abacus Concepts, Statview package (version 4.1) for the Apple MacIntosh (Abacus Concepts, Inc., Berkeley, CA, USA).

Experiment 2: Individual hormone profiles during the baseline and treatment periods were analysed using a computer pulse detection program (Munro, Zaristow software, Haddington, Scotland) as previously described (Brooks and Challis 1991). Briefly, the program generates a baseline by examining local nadirs, and then detects pulses as deviations from this baseline using preset parameters. A moving average of 100 min was used to determine baseline hormone concentrations, and values of two standard deviations or more from the previous nadir were considered to be significant pulses. The interpulse interval and pulse amplitude during baseline and treatment periods in control and treated animals were compared by ANOVA.

Because absolute ACTH and cortisol concentrations varied between animals in later gestation (reflecting earlier activation of the HPA axis in some animals) it was necessary to normalise the data for comparison of mean levels between groups. The same analysis was applied for GH concentrations. For each individual animal ACTH, cortisol and GH concentrations were expressed as a percentage of the mean concentration during the 3 h basal sampling period. The group means were then calculated and treatment and control groups compared by two factor ANOVA with post hoc Scheffe's test. The response to NMDA challenge at the end of the serial sampling period was similarly assessed by normalising hormone concentrations to the average concentration in the preceding 30 min.

3.4 Results

Gestation changes in basal hormone concentrations

All animals had measurable quantities of ACTH at 130 and 138 days of gestation. As was expected there was a significant increase in mean basal ACTH concentration between day 130 and day 138. The mean basal ACTH (all animals) at day 130 was 16.60±7.37 pg/ml and at day 138 was 107.47±43.68 pg/ml (t-test; $p<0.01$). Similarly, GH could be detected at both 130 and 138 days gestation and increased significantly with gestation. Mean basal levels were 12.8±3.43 ng/ml at 130 days and 55.96±14.82 ng/ml at 138 days (t-test; $p<0.01$).

Effect of NMDA receptor antagonism on insulin, sodium nitroprusside and NMDA stimulated ACTH and GH secretion

Insulin administration on day 130 gestation stimulated a significant increase in ACTH concentration, which was attenuated by CGP 37849 pretreatment (ANOVA; Change with time $p<0.0001$, Treatment by time interaction $p<0.02$). In contrast, sodium nitroprusside infusion resulted in a profound increase in circulating ACTH concentrations, peaking at 15 min, that was not significantly affected by pretreatment with CGP 37849. The ACTH responses to insulin and sodium nitroprusside are shown in figure 3.1. Insulin induced hypoglycaemia resulted in a modest but significant decline in GH levels that was unaffected by CGP 37849 (ANOVA; Change with time $p<0.0001$, Treatment by time interaction $p>0.05$). Sodium nitroprusside was without effect on GH release. The GH responses to insulin and sodium nitroprusside are shown in figure 3.2. The ACTH response to NMDA administration was rapid and completely abolished by pretreatment with CGP 37849 at the dose used (ANOVA; Change with time $p<0.0001$, Treatment by time interaction $p<0.0001$). The ACTH response to NMDA is shown in figure 3.3.

Effect of NMDA receptor antagonism on pre-parturient ACTH, cortisol and GH secretion

On day 138 all animals displayed pulsatile ACTH, cortisol and GH secretion during the baseline period. The ACTH and cortisol concentrations varied between animals reflecting the fact that they were sampled at different time points during the endogenous prepartum increase in ACTH and cortisol secretion. For this reason ACTH and cortisol levels were expressed as a percentage of the mean concentration during the baseline period. After normalisation mean ACTH and cortisol concentrations in control animals increased with time over the duration of the sampling period. This may reflect a diurnal rhythm or a mild stress response to the sampling regime. There was a clear and highly significant suppression of ACTH and cortisol following the start of treatment with CGP 37849 (ANOVA; Treatment by time interaction $p<0.0001$ for ACTH and $p<0.0003$ for cortisol). The concentration of ACTH was depressed to a greater degree then that of cortisol. The changes in ACTH, cortisol and GH concentrations with CGP 37849 treatment are shown in figure 3.4. When ACTH pulse amplitude and interpulse interval were compared there were no differences between groups or with treatment (Table 1). Similarly, there were no differences in cortisol pulse amplitude or interpulse interval between groups or with treatment (Table 1). At the end of the 6 h baseline and treatment period, intravenous administration of NMDA evoked a robust and significant elevation of ACTH and cortisol concentration in control animals. This increase was not observed in animals receiving CGP 37849 (ANOVA; Treatment by time interaction $p<0.01$ for ACTH and $p<0.01$ for cortisol). The ACTH, cortisol and GH responses to NMDA challenge at the end of the infusion period are shown in figure 3.5.

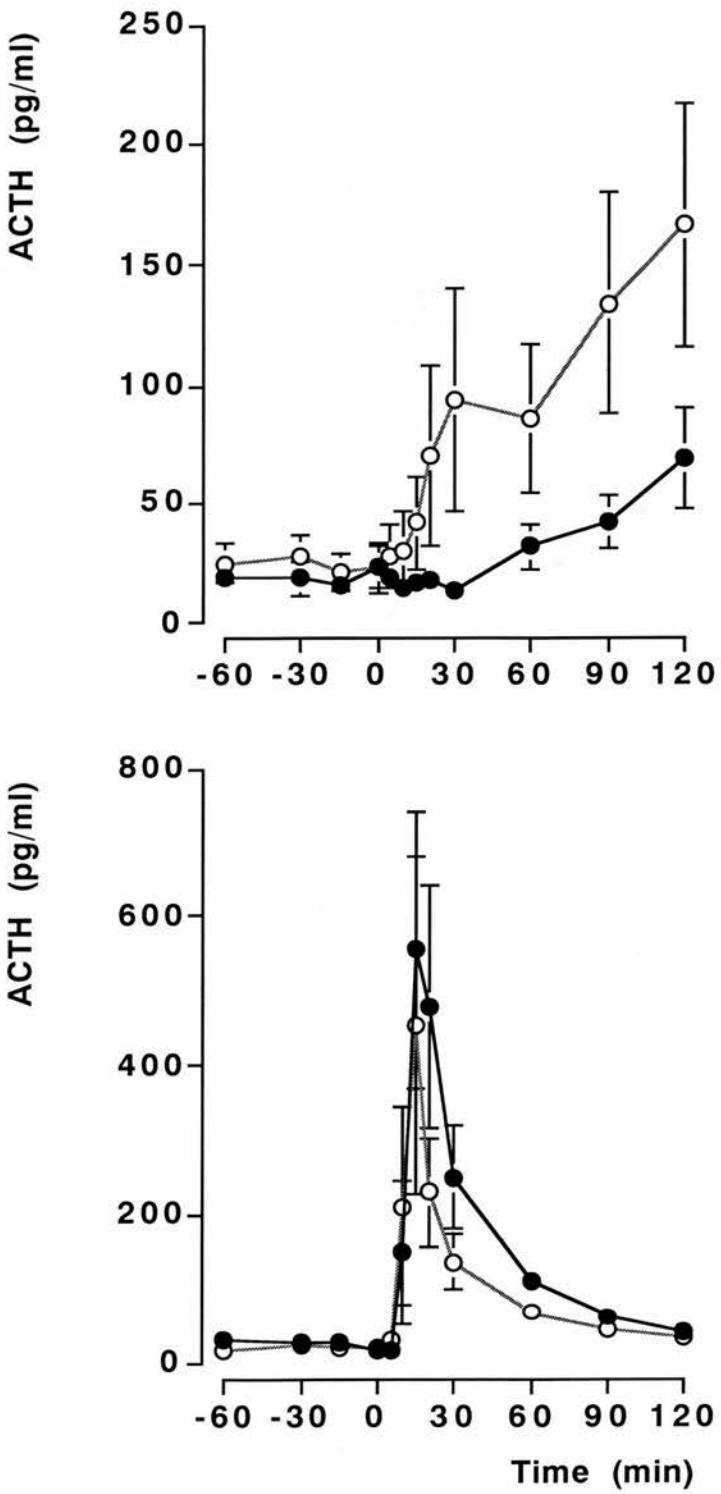
In contrast, CGP 37849 had no effect on GH concentrations, and there were no differences in GH pulse amplitude or interpulse interval between groups or with treatment (Table 1). There was, however, a significant increase in GH concentration in response to the NMDA challenge in the control group, which was abolished in the animals receiving CGP 37849 (ANOVA; Treatment by time interaction $p<0.01$).

Overleaf

Figure 3.1

Effect of CGP 37849 on the ACTH response to challenge with insulin (10 IU iv bolus) (upper panel) or sodium nitroprusside (100 μg/min iv infusion for 10 min) (lower panel). Fetuses were pretreated with CGP37849 (1 mg/kg iv bolus) (n=5, filled circles) or saline vehicle (n=5, open circles) and the challenge administered 5 min later. Values are mean±SEM. Pretreatment with CGP 37849 significantly attenuated the ACTH response to insulin induced hypoglycaemia (ANOVA; Effect of time $p<0.0001$, Treatment by time interaction $p<0.02$) but not to sodium nitroprusside (ANOVA; Effect of time $p<0.0001$, Treatment by time interaction $p>0.05$).

3.1

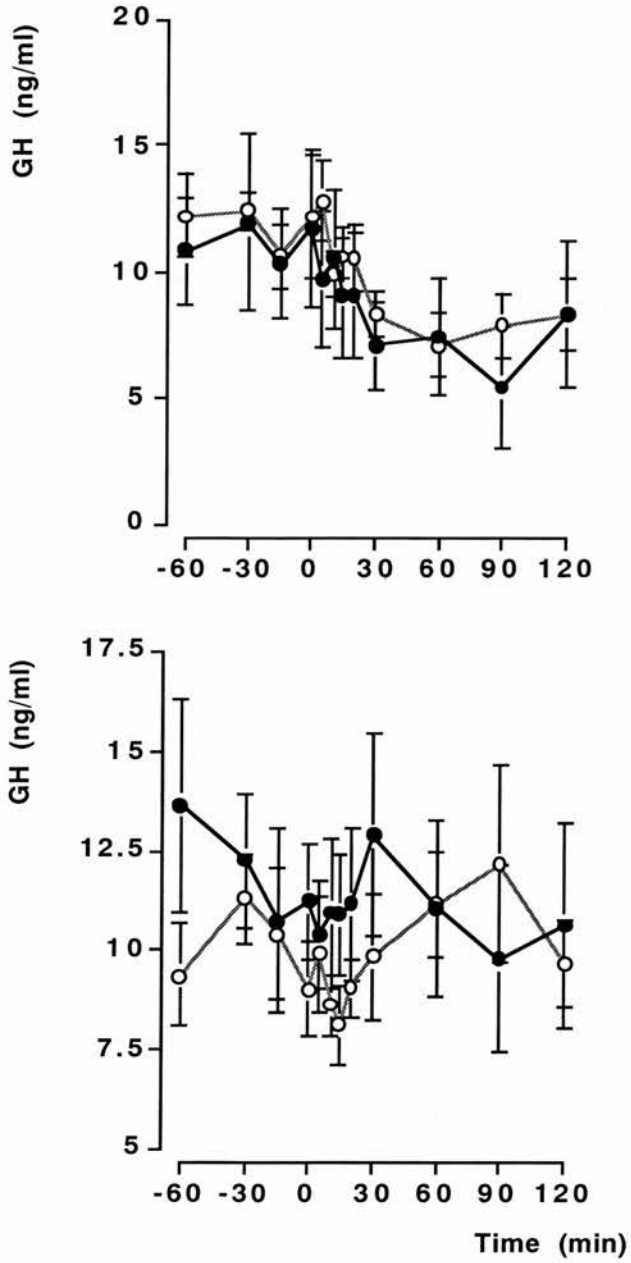

ACTH (pg/ml)
250
200
150
100
50
0
-60 -30 0 30 60 90 120
ACTH (pg/ml)
800
600
400
200
0
-60 -30 0 30 60 90 120
Time (min)

Overleaf

Figure 3.2

Effect of CGP 37849 on the GH response to challenge with insulin (10 IU iv bolus) (upper panel) or sodium nitroprusside (100 μg/min iv infusion for 10 min) (lower panel). Fetuses were pretreated with CGP37849 (1 mg/kg iv bolus) (n=5, filled circles) or saline vehicle (n=5, open circles) 5 min before the challenge was administered. Values are mean±SEM. Insulin induced hypoglycaemia caused a significant decline in GH concentration that was unaffected by pretreatment with CGP 37849 (ANOVA; Change with time $p<0.0001$, Treatment by time interaction $p>0.05$). Sodium nitroprusside treatment was without effect on GH concentrations.

3.2

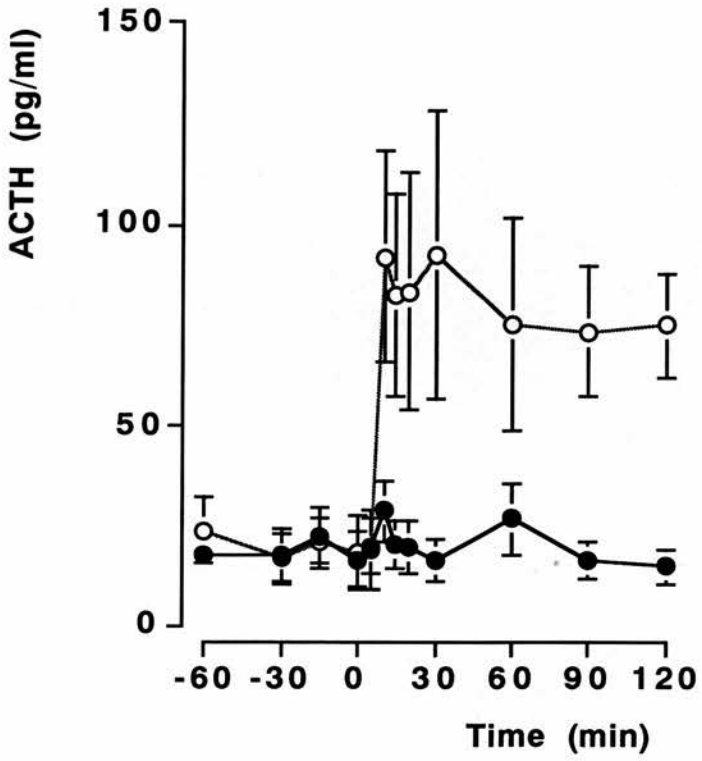

GH (ng/ml)
20
15
10
5
0
-60 -30 0 30 60 90 120
GH (ng/ml)
17.5
15
12.5
10
7.5
5
-60 -30 0 30 60 90 120
Time (min)

Overleaf

Figure 3.3

Effect of CGP 37849 on the ACTH response to challenge with NMDA (4 mg/kg iv bolus). Fetuses were pretreated with CGP 37849 (1 mg/kg iv bolus) (n=5, filled circles) or saline vehicle (n=5, open circles) 5 min before the challenge was administered. Values are mean$\pm$SEM. The ACTH response to NMDA was significantly inhibited by CGP 37849 (ANOVA; Effect of time $p<0.0001$, Treatment by time interaction $p<0.0001$).

3.5

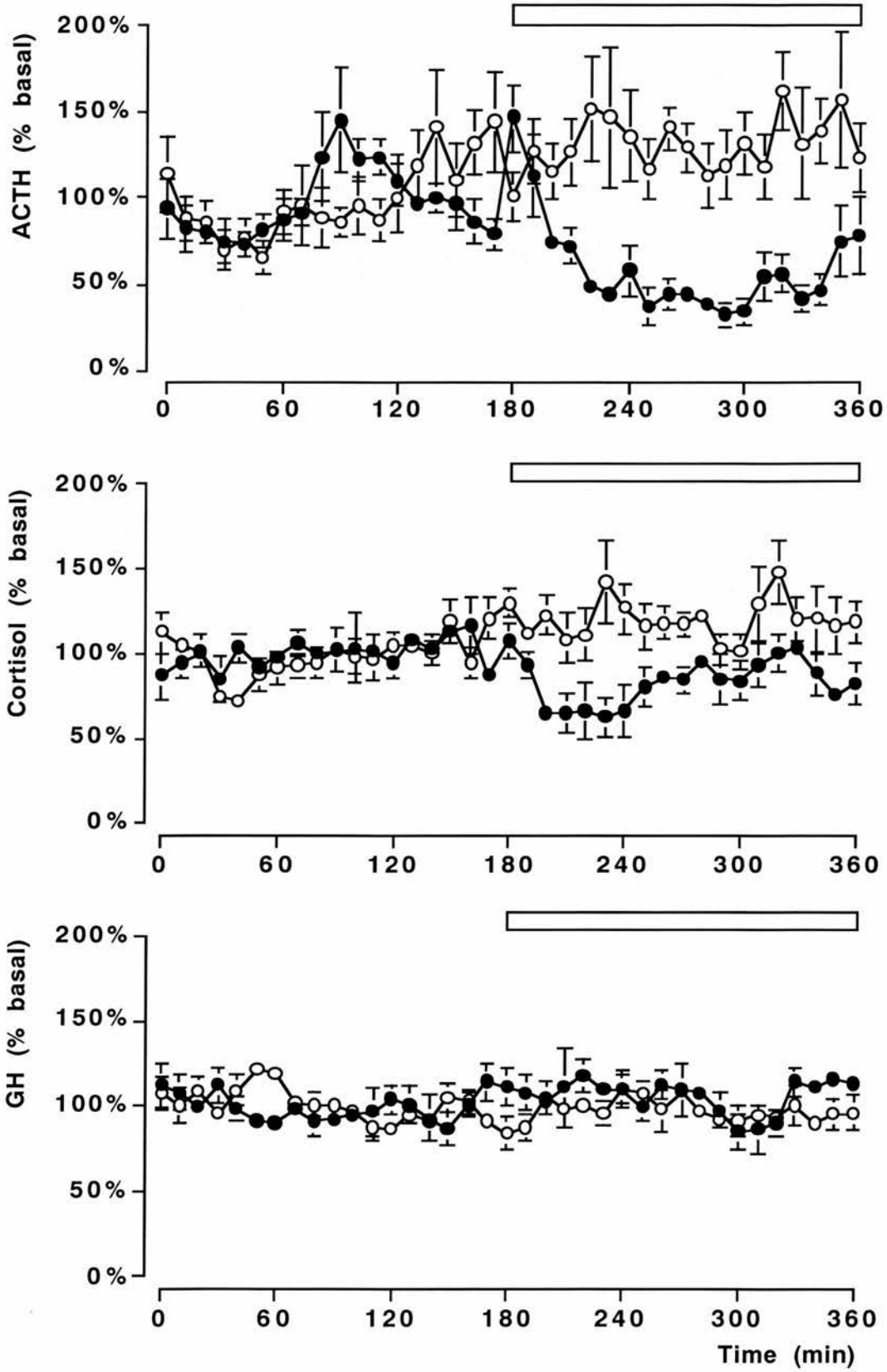


Overleaf

Figure 3.4

Effect of CGP 37849 on plasma ACTH (upper panel), cortisol (middle panel) and GH (bottom panel) concentrations (expressed as percentage change from mean baseline concentrations) at day 138 gestation. After 3 h baseline sampling fetuses received a bolus of CGP37849 (1 mg/kg iv) followed by an infusion of CGP37849 (1 mg/kg/h) for a further 3 h (n=5, filled circles) or saline vehicle (n=6, open circles). Values are mean$\pm$SEM. Infusion of CGP 37849 significantly reduced ACTH and cortisol concentrations (ANOVA; Treatment by time interaction $p<0.0001$ for ACTH and $p<0.0003$ for cortisol), but not GH concentrations (Treatment by time interaction $p>0.05$).

3.4

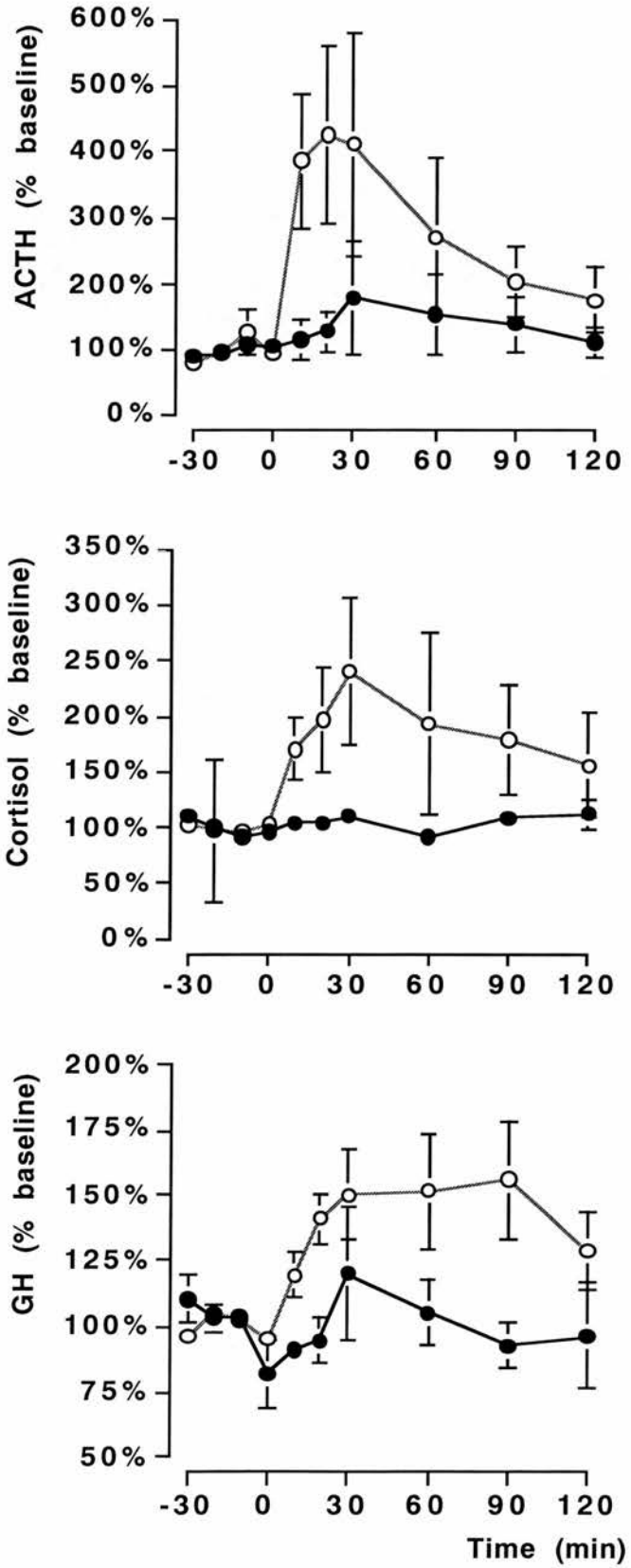

ACTH (% basal)
200%
150%
100%
50%
0%
0
60
120
180
240
300
360
Cortisol (% basal)
200%
150%
100%
50%
0%
0
60
120
180
240
300
360
GH (% basal)
200%
150%
100%
50%
0%
0
60
120
180
240
300
360
Time (min)

Overleaf

Figure 3.5

Inhibition of ACTH (upper panel), cortisol (middle panel) and GH (bottom panel) responses to NMDA by CGP 37849 infusion. After 3 h of infusion of CGP 37849 (n=5, filled circles) or saline (n=6, open circles), fetuses were challenged with NMDA (4 mg/kg iv bolus) and the infusion of CGP 37849 (1 mg/kg/h) continued. Hormone concentrations are expressed as a percentage of mean over the preceding 30 min baseline. Values are mean $\pm$SEM. Challenge with NMDA produced an elevation of plasma ACTH, cortisol and GH concentrations that was significantly inhibited by CGP 37849 infusion (ANOVA; Treatment by time interaction $p<0.01$ for ACTH, $p<0.01$ for cortisol and $p<0.01$ for GH).

3.5

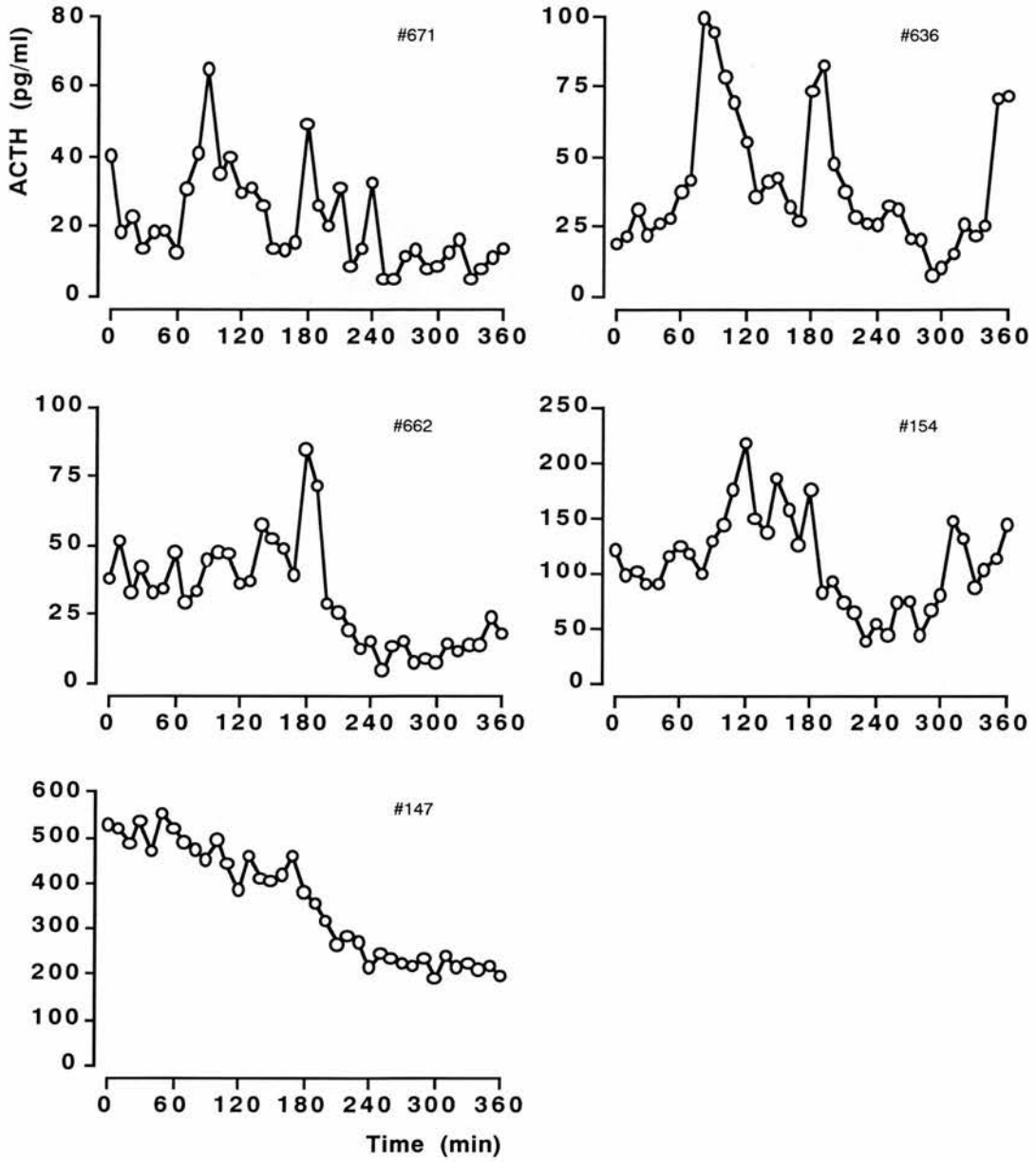

ACTH (% baseline)
600%
500%
400%
300%
200%
100%
0%
-30
0
30
60
90
120
Cortisol (% baseline)
350%
300%
250%
200%
150%
100%
50%
0%
GH (% baseline)
200%
175%
150%
125%
100%
75%
50%
Time (min)

Overleaf

Figure 3.6

Montage showing plasma ACTH concentrations in individual animals receiving CGP 37849. The NMDA antagonist was given as a bolus of 1 mg/kg after 180 min and continued as an infusion (1 mg/kg/h) for the remainder of the sampling period.

3.6

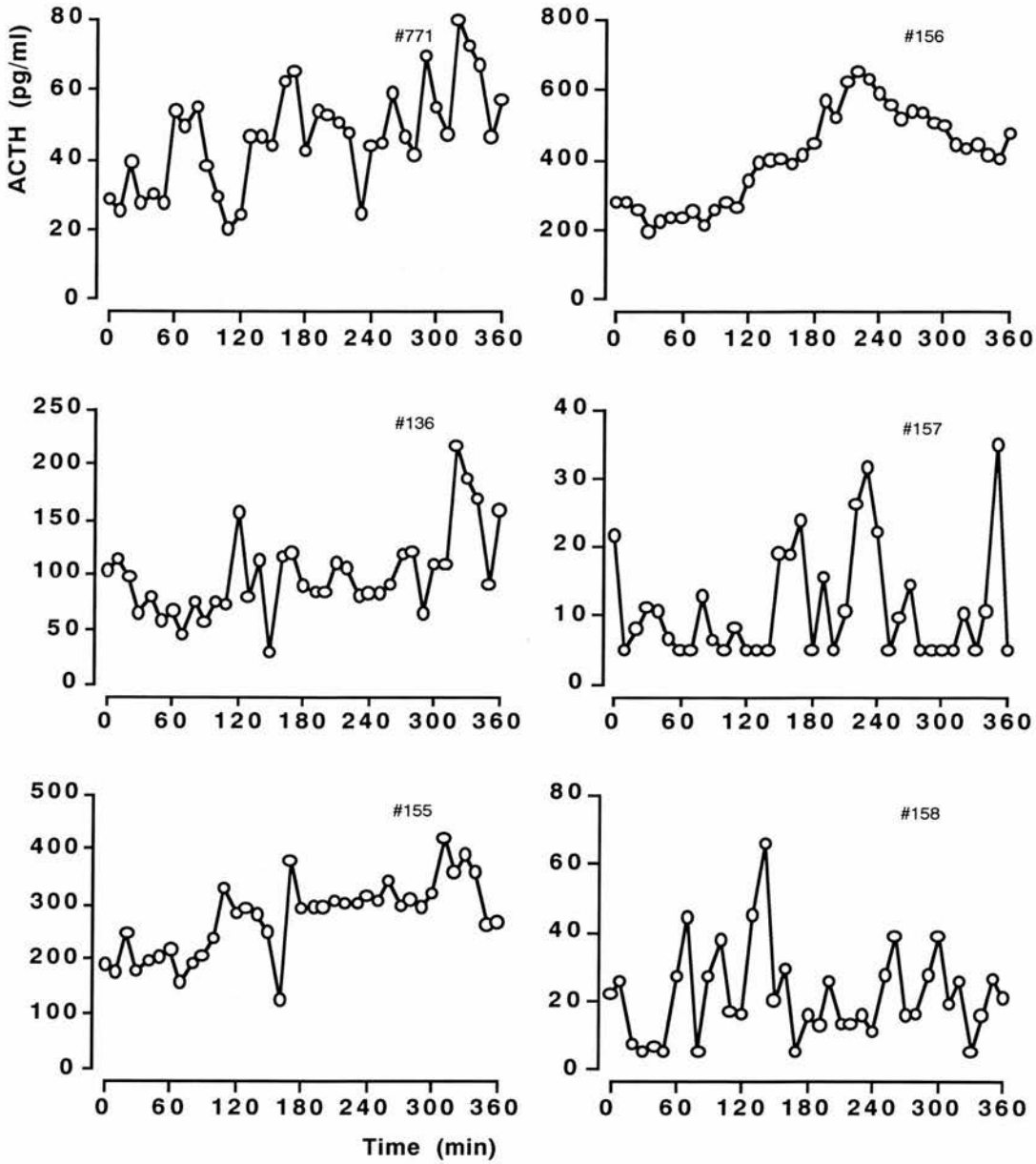


Overleaf

Figure 3.7

Montage showing plasma ACTH concentrations in individual animals receiving saline vehicle. The saline was given as a bolus of 2.5 ml after 180 min and continued as an infusion of 2.5 ml/h for the remainder of the sampling period.

3.7

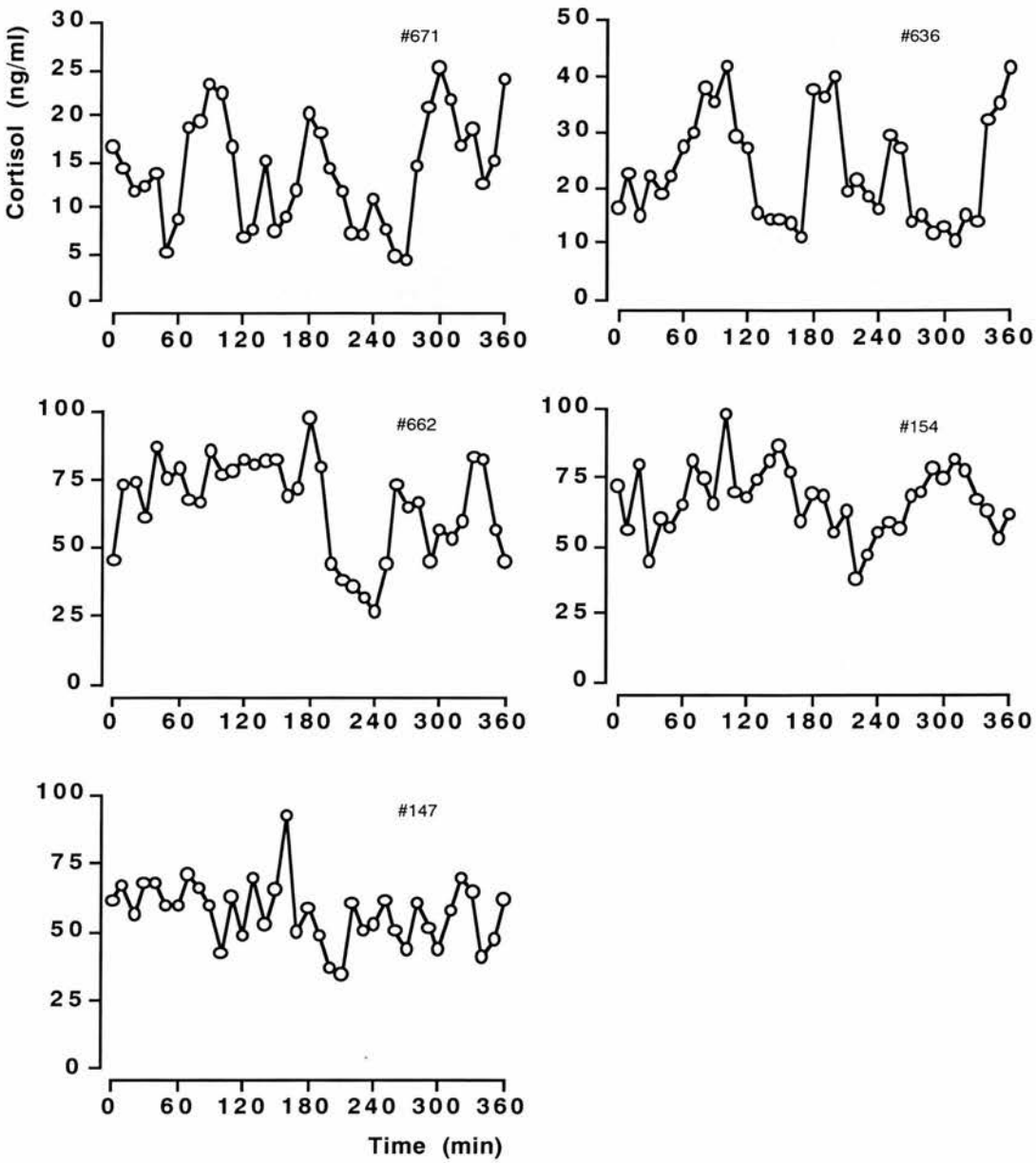


Overleaf

Figure 3.8

Montage showing plasma cortisol concentrations in individual animals receiving CGP 37849. The NMDA antagonist was given as a bolus of 1 mg/kg after 180 min and continued as an infusion (1 mg/kg/h) for the remainder of the sampling period.

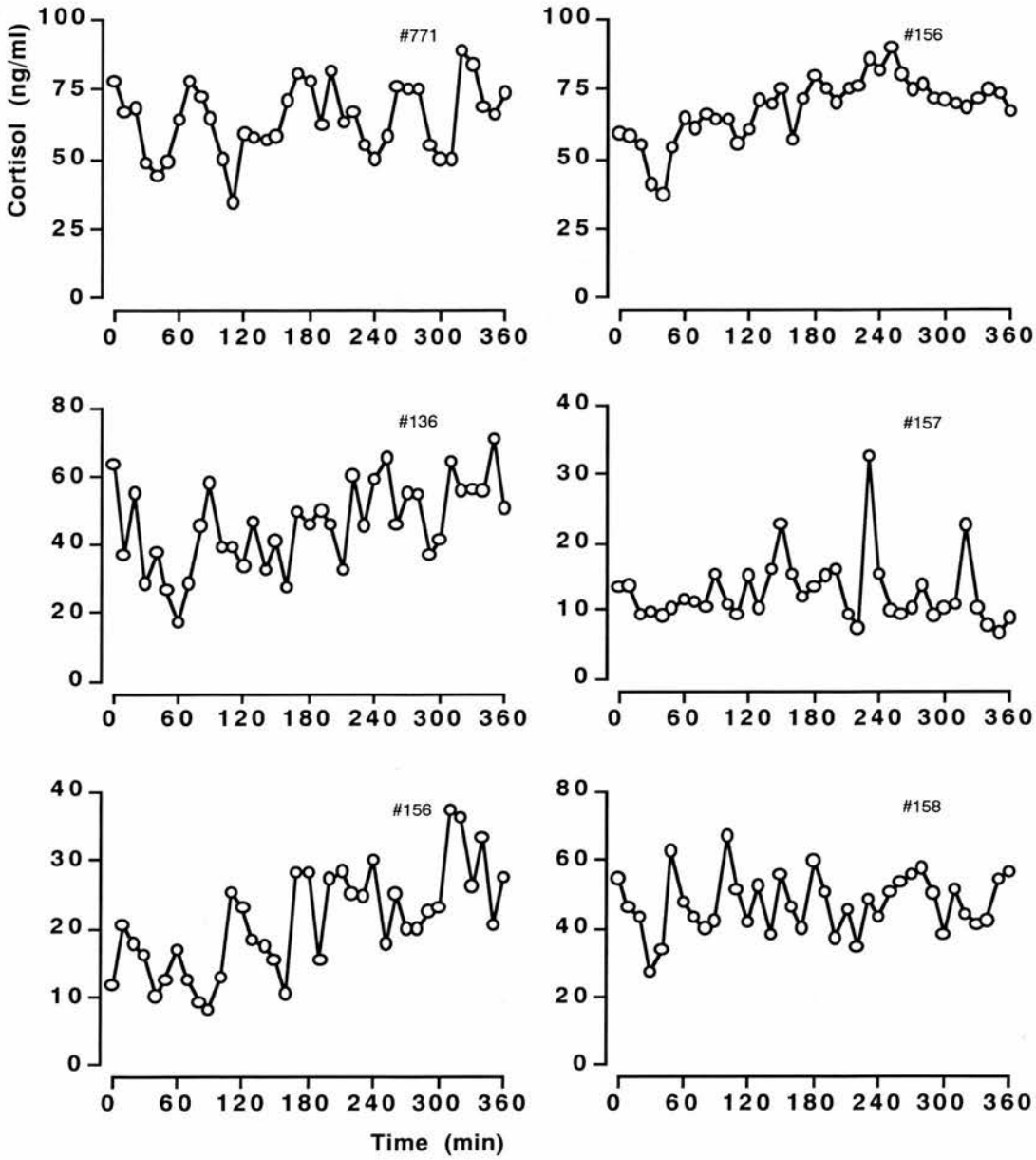
3.8


Cortisol (ng/ml)
#671
#636
#662
#154
#147
Time (min)
0
60
120
180
240
300
360

Overleaf

Figure 3.9
Montage showing plasma cortisol concentrations in individual animals receiving saline vehicle. The saline was given as a bolus of 2.5 ml after 180 min and continued as an infusion of 2.5 ml/h for the remainder of the sampling period.

3.9

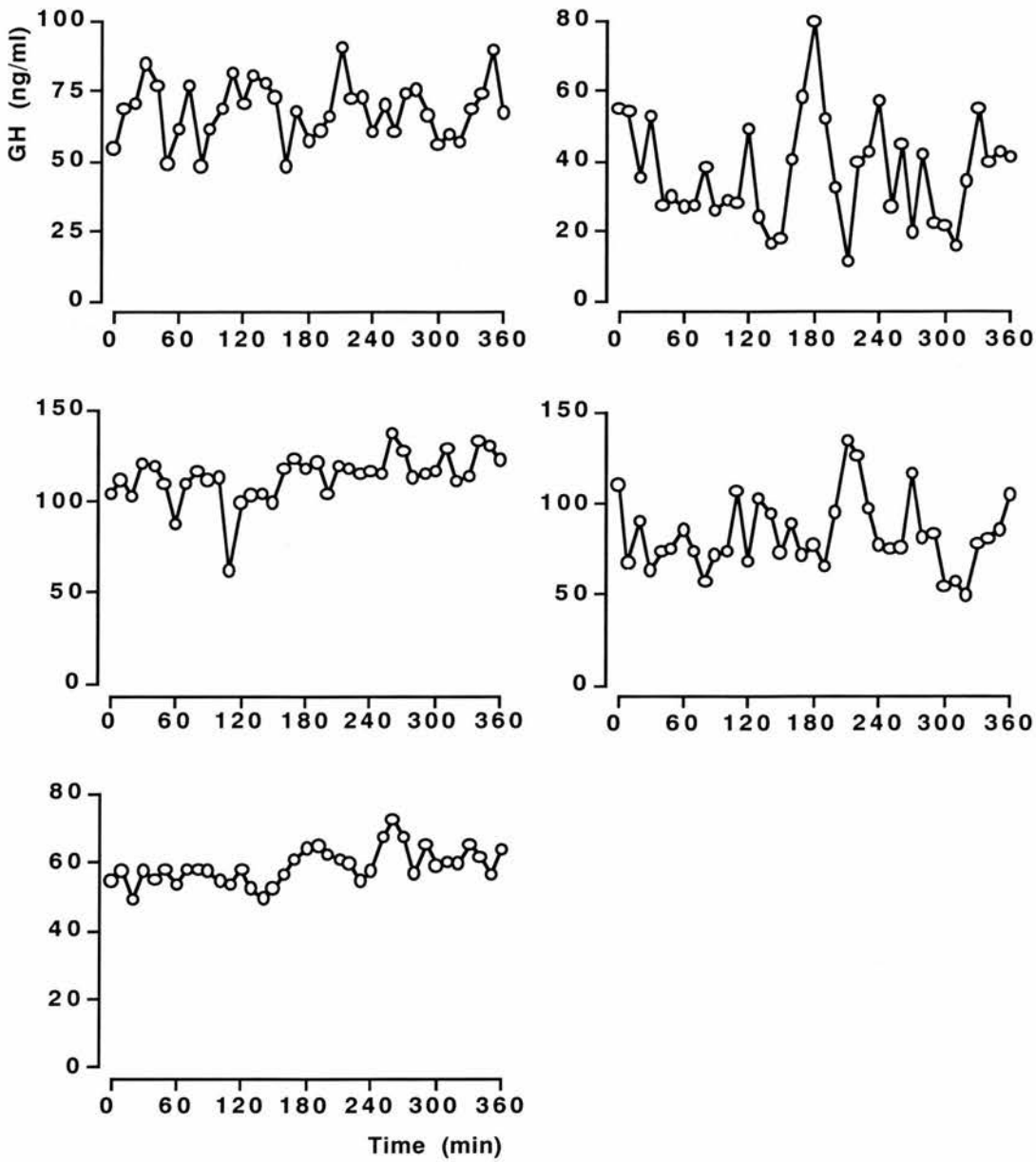


Overleaf

Figure 3.10

Montage showing plasma GH concentrations in individual animals receiving CGP 37849. The NMDA antagonist was given as a bolus of 1 mg/kg after 180 min and continued as an infusion (1 mg/kg/h) for the remainder of the sampling period.

3.10


GH (ng/ml)
100
75
50
25
0
80
60
40
20
150
0 60 120 180 240 300 360
Time (min)

Overleaf

Figure 3.11

Montage showing plasma GH concentrations in individual animals receiving saline vehicle. The saline was given as a bolus of 2.5 ml after 180 min and continued as an infusion of 2.5 ml/h for the remainder of the sampling period.

3.11

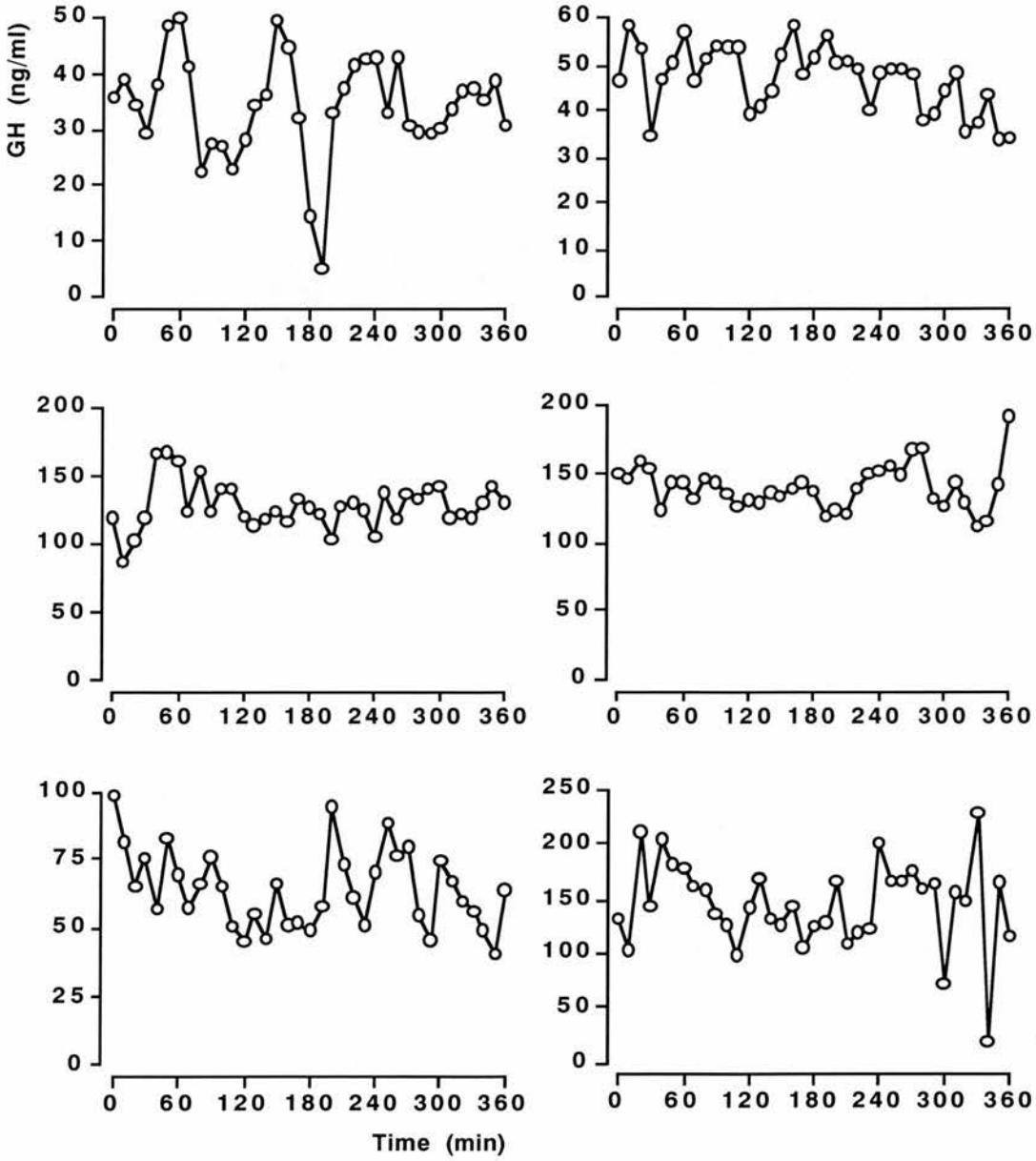


Table 1: Effects of NMDA receptor antagonism with CGP 37849 on pulsatile characteristics of ACTH, GH and cortisol secretion.

Pulse amplitude (mean±SEM)

		Basal period	Treatment period
ACTH	CGP 37849	38.7±5.4	25.2±6.8
(pg/ml)	Saline	34.3±7.2	34.8±8.6
GH	CGP 37849	22.4±4.6	30.2±6.6
(ng/ml)	Saline	17.2±2.1	20.9±3.6
Cortisol	CGP 37849	12.6±5.4	16.4±3.2
(ng/ml)	Saline	16.8±3.0	18.4±2.6

Interpulse interval (min) (mean±SEM)

		Basal period	Treatment period
ACTH	CGP 37849	54.4±9.3	45.0±5.6
	Saline	42.2±10.0	46.0±7.0
GH	CGP 37849	39.0±4.1	41.1±5.8
	Saline	41.6±5.8	42.9±5.5
Cortisol	CGP 37849	40.0±10.0	60.0±5.5
	Saline	48.8±6.7	45.0±5.3

3.5 Discussion

A significant role for endogenous excitatory amino acid neurotransmitters acting at the NMDA receptor to mediate basal activity of the fetal HPA axis, and its response to certain stresses, is demonstrated in these experiments. At day 130 gestation pretreatment with CGP 37849 inhibited the rise in ACTH concentration seen in response to insulin-induced hypoglycaemia indicating a role for NMDA receptors in this stress responsive pathway. Later in gestation, at a time when basal (unstimulated) ACTH concentrations are increased in preparation for parturition, treatment with CGP 37849 resulted in a decline in mean ACTH and cortisol concentrations. In contrast GH secretion was unchanged by NMDA antagonism.

At 130 days gestation ACTH secretion is readily stimulated by NMDA as previously reported (Brooks and Howe 1996). The compound CGP 37849 has been shown to be a competitive NMDA receptor antagonist in the rat (Schmutz, Portet et al. 1990), and the demonstration that pretreatment with CGP 37849 completely abolishes the ACTH response to NMDA confirms that CGP 378494 is an NMDA antagonist in the ovine fetus. Using CGP 37849 the role of endogenous excitatory amino acids acting at the NMDA receptor in the regulation of ACTH secretion has been examined.

Insulin-induced hypoglycaemia stimulated ACTH secretion in the ovine fetus, and this is in agreement with earlier work (Ozolins, Young et al. 1992). There is good evidence in adult animals that both AVP and CRH are involved in this response. Direct sampling of portal plasma in conscious adult sheep during insulin induced hypoglycaemia revealed that AVP and CRH are secreted in increased amounts (Engler, Pham et al. 1989; Caraty, Grino et al. 1990), and chronic active immunisation against AVP or CRH diminished the ACTH response in adult sheep (Guillaume, Conte-Devolx et al. 1992; Guillaume, Conte-Devolx et al. 1992). The involvement of the NMDA receptor pathways in this response has not been investigated, but is indicated by the attenuated ACTH response in the fetuses that were pretreated with CGP 37849.

A nitroprusside infusion was originally included in this study to examine the effects of NMDA receptor antagonism on the ACTH response to nitroprusside induced hypotension. Subsequently it has become apparent that nitroprusside, by acting as a nitric oxide donor, is able to bypass blockade of the NMDA receptor. Nitric oxide is a component of the signalling pathway generated after activation of the NMDA receptor in the luteinising hormone (LH) response to NMDA (Mahachoklertwattana, Black et al. 1994; Pu, Xu et al. 1996). Evidence from *in vitro* studies also supports a role for nitric oxide in the modulation of ACTH secretion (Costa, Poma et al. 1996) and neurons of the PVN and SON are immunoreactive for both NMDAR1 and nitric oxide synthase (Bhat, Mahesh et al. 1995).

In the late gestation ovine fetus two studies have identified pulsatile secretion of ACTH but were unable to demonstrate any change in pulse amplitude or frequency in the few days before labour (Brooks and Challis 1991; Apostolakis, Longo et al. 1992). Our data on pulse frequency and amplitude are in broad agreement with this earlier work. We find that pulses are variable in amplitude and duration and that the mean ACTH concentrations vary considerably between animals. This presumably reflects the fact that fetuses were sampled at different time points during the pre-parturient ACTH surge. The administration of CGP 37849 resulted in a rapid decline in mean ACTH concentrations but was without significant effect on pulse frequency and amplitude. This suggests that ACTH secretion comprises two components: basal secretion and superimposed pulsatile secretion, and that NMDA receptors are important for basal secretion.

A recent study reports the persistence of pulsatile ACTH secretion in the hypothalamo-pituitary disconnected ovine fetus (Canny, Young et al. 1998). This suggests that pulsatile secretion of ACTH is an intrinsic property of the pituitary and not dependent upon hypothalamic releasing factors. In adult animals, however, simultaneous sampling of portal blood and the systemic circulation reveals a high degree of synchrony between pulses of CRH and AVP and ACTH (Caraty, Griro et al. 1988; Engler, Pham et al. 1989). It is notable that both NMDA antagonism in this study and opioid receptor antagonism with naloxone (Brooks and Challis 1991) reduced mean

ACTH levels seemingly without affecting pulse characteristics. One explanation may be that hypothalamic releasing factors determine overall basal release and help synchronise secretion by the corticotrophs, but that pulses of releasing factor are not obligatory for secretion of ACTH. In support of this it is found that a significant number of corticotrophs in culture secrete ACTH in the absence of CRH or AVP, but exposure to the releasing hormones changes the electrical activity of the corticotroph and increases release of ACTH (Kwiecieu and Hammond 1998). An alternative explanation is that ACTH pulses are intrinsically variable in amplitude and frequency, and that the limitations of the ACTH assay and pulse detection analysis conspire to hide subtle changes in pulse characteristics. Interestingly, LH pulses, which generally have more consistent secretory characteristics, are inhibited by NMDA antagonism which has an effect to reduce pulse amplitude without affecting pulse frequency (Ping, Mahesh et al. 1995).

The decline in ACTH concentration during infusion of CGP 37849 also results in a decrease in circulating cortisol concentrations. The magnitude of the decrease, however, is less than that of ACTH. Inspection of hormone profiles shows a remarkable concordance between ACTH and cortisol in some animals, implying very acute modulation of cortisol secretion by ACTH. In other animals, however, cortisol pulses seem to continue despite a marked drop in circulating ACTH concentration. Others report a dissociation between ACTH and cortisol pulses in the ovine fetus (Apostolakis, Longo et al. 1992). It may be that the adrenal is actually responding to small changes in ACTH concentration, rather than absolute levels, and that the techniques used in the present study have not been sufficiently sensitive to detect small fluctuations in ACTH concentration. The fetal adrenal gland certainly does not need pulsatile ACTH for there to be an increase in cortisol secretion since infusions of low doses of ACTH into hypophysectomised fetuses result in increased cortisol secretion (Jacobs, Young et al. 1994).

The secretion of GH in late gestation ovine fetuses is also pulsatile (Bassett and Gluckman 1986; Bauer, Breier et al. 1995). Reported pulse frequencies are somewhat lower than ours (0.74 ± 0.46 pulses/h (Bassett and Gluckman 1986)), but this may be a consequence of different sampling

frequencies and pulse detection programmes. Our data on pulse amplitude and basal (nadir) levels are within the range previously documented (Bassett and Gluckman 1986; Bauer, Breier et al. 1995). In contrast to the situation with ACTH, antagonism of NMDA receptors is without effect on mean GH concentrations or pulse amplitude or frequency, though GH is raised in response to NMDA challenge in the control animals. The difference in the responses of ACTH and GH to NMDA and CGP 37849 may be explained by differences in the control of their secretion from the anterior pituitary. Hypothalamic control of GH secretion is mediated by the stimulatory action of growth hormone releasing hormone (GHRH) and the inhibitory action of somatostatin. Pulses of GH secretion in the adult animal are generated by a simultaneous decline in somatostatin secretion and an increase in GHRH secretion (Plotsky and Vale 1985; Frohman, Downs et al. 1990). It is possible that NMDA antagonism reduces both GHRH and somatostatin secretion so that the overall drive to the somatotroph is unchanged. Several studies have confirmed that exogenous NMDA stimulates GH secretion (Estienne, Schillo et al. 1989; Acs, Lonart et al. 1990; Downing, Joss et al. 1996). This action of NMDA is thought to be via increased GHRH secretion (Brann and Mahesh 1997). For instance, NMDA stimulated GH secretion is prevented by destruction of GHRH neurons in the arcuate nucleus and by pretreatment with GHRH antibody (Acs, Lonart et al. 1990). Chronic NMDA antagonism reduces hypothalamic GHRH mRNA but not somatostatin mRNA (Cocilovo, Colonna et al. 1992). At the same time, release of somatostatin from hypothalamic explants in response to hypoglycaemia is NMDA dependent (Issa, Lewis et al. 1998) and direct measurement of somatostatin secretion at the median eminence demonstrates a role for NMDA sensitive pathways in stimulating somatostatin release (Estupina, Abarca et al. 1996).

An important role for endogenous neurotransmitters acting at the NMDA receptor in the late gestation activation of the fetal HPA axis is indicated by the acute decline in mean ACTH concentrations following administration of NMDA antagonist. This suggests that NMDA sensitive pathways are involved in initiating the endocrine events that lead to labour in the sheep. Most evidence indicates that NMDA receptor agonists influence

anterior pituitary hormone secretion via an action within the hypothalamus. NMDA receptors have been demonstrated by autoradiography, in-situ hybridization and by immunocytochemistry in the central nervous system and also in lower abundance in the pituitary gland in rodents (Bhat, Mahesh et al. 1995).

The secretion of ACTH from the anterior pituitary is controlled principally by AVP and CRH secreted by parvocellular neurons located in the PVN. NMDA receptors are located within the PVN and administration of glutamate directly into the PVN stimulates ACTH release (Darlington, Miyamoto et al. 1989). Several studies have documented the involvement of CRH in the response to NMDA. Administration of glutamate into the amygdala stimulates release of CRH at the median eminence in a dose responsive manner (Gabr, Birkle et al. 1995). In the immature rat NMDA stimulates the release of ACTH and this action can be blocked by anti-CRH antibodies (Chautard, Boudouresque et al. 1993). In adult rat hypothalami, however, NMDA stimulates the release of AVP but not CRH (Costa, Yasin et al. 1992; Patchev, Karalis et al. 1994) though others report an effect on CRH secretion (Joanny, Steinberg et al. 1997). The principal anatomical site at which endogenous excitatory amino acids act on the NMDA receptor to regulate ACTH secretion in the ovine fetus is unclear. Recently it has been suggested that exogenous NMDA stimulates ACTH secretion through a direct action at the pituitary in the ovine fetus (Szeto, Soong et al. 1999) since the ACTH response to NMDA could not be inhibited by pretreatment with an AVP or CRH antagonist.

The unique properties of the NMDA receptor enable it to play important roles in synaptic plasticity and the organisation of the developing brain. Activation of the NMDA receptor is thought to stabilise developing synaptic connections and inhibit neuronal apoptosis (Wu, Malinow et al. 1996; Zhang, Rubin et al. 1998). In the developing cerebellum and hippocampus the NMDA receptor subtype expressed is developmentally regulated and correlates to the appearance of a mature pattern of nerve activity (Farrant, Feldmeyer et al. 1994; Gottmann, Mehrle et al. 1997). Blockade of the NMDA receptor disrupts the normal elimination of excess

synapses in the cerebellum (Rabacchi, Bailly et al. 1992). Similar events probably occur during development of the neuroendocrine hypothalamus. For example, at puberty there is a reduction in the number of synapses onto LHRH neurons (Perera and Plant 1997). In the female rat there is a critical period for the action of NMDA to advance puberty which may relate to development of the neuronal circuitry controlling LHRH release (Smyth and Wilkinson 1994). There is conflicting evidence about the level of expression of NMDA receptors in the hypothalamus over this time period (Nyberg, Srivastava et al. 1995; Eyigor and Jennes 1997). The NMDA receptor may have a comparable influence on the functional maturation of the HPA axis during critical periods in fetal development and as such would offer a molecular locus determining the timing of parturition in the sheep.

In summary, these studies have demonstrated that excitatory amino acid neurotransmitters acting through the NMDA receptor mediate basal activity of the HPA axis in the immediate period leading to parturition, and also the response to certain stresses. It is tempting to speculate that increasing fetal HPA activity in late gestation is associated with an increase in the number of NMDA receptors or a change in the pattern of subunit expression associated with the maturation of the neuronal circuitry controlling ACTH secretion.

4

Arginine vasopressin and corticotrophin releasing hormone regulation by central N-methyl-D-aspartate pathways : a microdialysis study

4.1 Abstract

Endogenous excitatory amino acid neurotransmitters acting through the NMDA receptor provide drive to basal ACTH secretion in the fetal sheep in late gestation, since the NMDA antagonist CGP 37849 reduces plasma ACTH concentrations. The principal site of action of CGP 37849, however, is unkown. A microdialysis technique was developed in the chronically cannulated unanaesthetised ovine fetus to investigate the effect of CGP 37849 on secretion of AVP and CRH at the median eminence. Insulin-induced hypoglycaemic stress was used to elevate AVP and CRH secretion. Fetal sheep between 125 and 130 days gestation were pretreated with CGP 37849 (1 mg/kg) or saline vehicle before iv challenge with 10 IU insulin. Plasma concentrations of ACTH and cortisol increased significantly following insulin challenge in control animals, and this increase was attenuated in animals that had been pretreated with CGP 37849. Insulin-induced hypoglycaemia stimulated an increase in the concentration of AVP recovered in median eminence dialysate that was unaffected by pretreatment with CGP 37849. These results demonstrate that AVP secretion at the median eminence is not regulated by NMDA receptor coupled pathways. Unfortunately, no CRH could be detected in any of the dialysis samples.

Median eminence dialysate also contained measurable, but low quantities of glutamate, but concentrations were unaffected by insulin-induced hypoglycaemia.

4.2 Introduction

An important role for endogenous excitatory amino acid neurotransmitters acting through the NMDA receptor to drive ACTH secretion in late gestation was demonstrated in chapter 3. The pathways through which NMDA stimulates ACTH secretion are not known. In situ hybridisation studies have colocalised CRH and the NMDA receptor in the PVN in the rat (Aubry, Bartanusz et al. 1996). Magnocellular AVP neurons also express the NMDA receptor (Decavel and Curras 1997). In the immature rat NMDA stimulated ACTH release is blocked by anti-CRH antibodies (Chautard, Boudouresque et al. 1993). Studies *in vitro* with adult rat hypothalami or slices are conflicting, with some reporting that NMDA stimulates the release of AVP but not CRH (Costa, Yasin et al. 1992), while others find that NMDA does stimulate CRH secretion (Joanny, Steinberg et al. 1997). The stimulation of ACTH by NMDA in the sheep fetus may even involve a direct action on the pituitary (Szeto, Soong et al. 1999). In an attempt to characterise the pathways through which the NMDA receptor controls ACTH secretion in the fetus a microdialysis technique is developed to sample the secretion of releasing factors at the median eminence in the chronically catheterised fetus.

Microdialysis was developed to allow the estimation of local neurotransmitter release in brain tissue (Benveniste and Huttemeier 1990; Stamford 1992; Westerink 1995). A length of tubing made from a semi-permeable membrane is implanted in the region of interest and perfused with a physiological solution. Neurotransmitters in the vicinity diffuse across the membrane in proportion to their local tissue concentration and membrane permeability and can then be measured in the collected dialysate if an assay of sufficient sensitivity is available. The technique has been used to measure glutamate release in the parietal cortex in the fetus during asphyxia (Tan, Williams et al. 1996), but has not been applied to the measurement of median

eminence peptide secretion. One of the difficulties is the accurate placement of the tip of the microdialysis probe in to the median eminence and this has necessitated a contrast guided stereotaxic approach.

4.3 Materials and Methods

Animals and surgical preparation

Mixed breed sheep with known single insemination dates were used in these experiments. Between day 125-130 gestation (term in our flock is 145 days) fetal sheep were prepared with chronic indwelling jugular, carotid and amniotic cannulae . A microdialysis probe was then positioned in the median eminence under X-ray guidance as described in section 2.1. Construction of the microdialysis probe is detailed below. To aid probe placement contrast medium was injected into the lateral ventricle via a lateral ventricle. Satisfactory placement of the microdialysis probe in the medium eminence was later confirmed by the recovery of AVP in the dialysate, and by visual inspection of the brain and pituitary at the completion of the experiment. Finally, emg electrodes (AS 632, Cooner Wire Co, Chatsworth, CA, USA) were sutured to the serosal surface of the myometrium.

On the afternoon of surgery animals were placed into metabolism crates and the microdialysis probe continuously perfused at 2 μl/min overnight with artificial cerebrospinal fluid (140 mM NaCl, 3 mM KCl, 2.4 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 0.27 mM NaH_2PO_4, 7.2 mM glucose pH7.4). Antibiotics were given to the fetus (10^6 units penicillin, Glaxovet, Uxbridge, UK) and mother (Streptopen, Glaxovet, Uxbridge, UK) for 3 days after surgery. Animals were fed twice daily and allowed free access to water. The cannulae were flushed daily with heparinised saline (20 IU/ml) and a small arterial sample withdrawn for blood gas analysis (IL1306; Instrumentation Laboratories, Warrington, Cheshire, UK). Only fetuses with maintained healthy acid-base status (measurements in close agreement with normal ranges of: pH 7.352±0.01; pO_2 20.28±0.49 mmHg; pCO_2 49.39±0.59 mmHg) were challenged with insulin-induced hypoglycaemia.

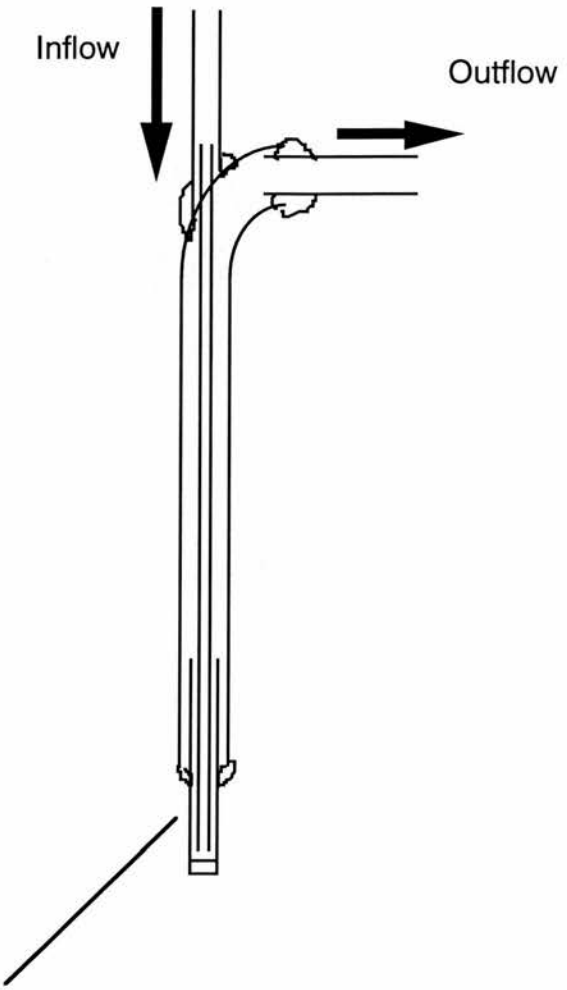
Microdialysis probes

Microdialysis probes were constructed from a length of 36g tubing (Coopers needle works, Birmingham, UK) placed concentrically inside a length of thin walled 24g tubing (Coopers Needle Works, Birmingham, UK). The tip of the 36g tubing protruded approximately 4 mm past the end of the outer tubing. A 10-15 mm length of dialysis membrane (Althin Medical Inc, Florida, USA) sealed at one end with epoxy resin was then pushed over the end of the 36g tubing, advanced inside the outer tubing and secured in position with more epoxy resin. At the other end of the assembly a short piece of polyvinyl tubing (Bolab V4, Bolab Inc., Lake Havasu City, USA) was cemented over the outer 24g tube and bent over to one side. The inner 36g tubing pierced the side wall of this polyvinyl tubing and protruded approximately 3 mm. A 100 cm length of polyvinyl tubing (Bolab V1, Bolab Inc., Lake Havasu City, USA) was attached to the end of the 36g tubing and served as the inflow channel. The outflow channel was created by cementing a 100cm length of polyvinyl tubing (FEP tubing, Carnegie Medicine, Stockholm, Sweden) into the Bolab V4 polyvinyl tubing. Both inflow and outflow channels were about 100cm long and were threaded through a larger diameter polyvinyl tube (Bolab V11, Bolab Inc., Lake Havasu City, USA) to prevent kinking. A diagrammatic representation of a microdialysis probe is given on the following page.

Recovery of neurotransmitters *in vitro*

The *in vitro* performance of the probe before implantation was assessed by dialysing a solution containing known amounts of AVP, CRH, glutamate and GABA overnight. The probe was perfused at 2 μl/minute as in the *in vivo* experiments with artificial csf. Mean (±SEM) in vitro recoveries were: AVP 7.2.4±2.1%, CRH 1.2±0.6%, glutamate 8.6±3% and GABA 5.2±4%.

Details of microdialysis system



The microdialysis probe is made up of two concentric steel tubes with a piece of dialysis tubing, sealed at one end with epoxy resin, cemented inside the outer tubing. Dialysis fluid forced down the inner tubing circulates past the dialysis membrane before passing back through the outer steel tubing to be collected.

Experimental design

To determine the role of endogenous excitatory amino acids acting at the NMDA receptor in the regulation of the hypothalamic releasing factors AVP and CRH, the secretion of these factors at the median eminence was examined by a microdialysis technique. Insulin induced hypoglycaemia was used as a challenge to elevate excitatory amino acid neurotransmitter activity after animals have been pretreated with CGP 37849 or saline vehicle. Differences in AVP or CRH release can then be attributed specifically to activation of the NMDA receptor. Because the performance of the microdialysis probe over time was not known these experiments were carried out 24 h after the probes were inserted. A second group of animals were also implanted with microdialysis probes and were dialysed continuously until they were adjudged to be in labour on the basis of myometrial emg activity. At this point an insulin challenge was administered.

Insulin-induced hypoglycaemia challenge

On the morning following surgery fetuses received an insulin challenge (10 IU iv bolus; Actrapid,Novo Nordisk, Crawley, UK) 5 minutes after pretreatment with either CGP37849 (4 mg iv bolus; Ceiba-Geigy, Basel, Switzerland) in 2 ml saline (n=5) or saline vehicle (n=5). Blood samples (1 ml sample, replaced with heparinised saline) were collected at -60, -30, 0, 10, 20, 30, 60, 90, and 120 min around the insulin injection. Microdialysis samples were collected over 30 min periods starting 60 min before insulin administration, allowing for the time delay in fluid reaching the collection tube. Samples were collected into 500 μl plastic tubes (Eppendorf, Hamburg, Germany), snap frozen on dry ice and stored at-20C until assay.

Long term performance of microdialysis system

A second group of 7 animals were prepared with microdialysis probes and dialysis fractions collected continuously every 2 h by automated fraction

collector until the sheep laboured. In addition, animals from the group challenged with insulin on the day after surgery were also followed into labour. Animals were inspected twice daily and blood samples withdrawn for hormone estimation. A blood gas analysis was also performed on each sample. Batches of accumulated dialysis samples were frozen every 12 h. A three way tap allowed replenishment of the csf reservoir without the incorporation of air bubbles into the dialysis probe. When the sheep were judged to be in labour by the appearance of an emg pattern characteristic of labour (Nathanielsz, Bailey et al. 1980; Farber, Guissani et al. 1997), a further insulin challenge (10 IU iv bolus) was administered. Samples were collected at -30, 0, 10, 20, 30, 60, 90, and 120 min around the sample where possible (in many animals in labour it was no longer possible to withdraw blood samples)

Radioimmunoassay

Samples were assayed in duplicate for immunoreactive ACTH $_{(1\text{-}39)}$ by a specific 2 site immunoradiometric assay (Brooks and Howe 1996) which is detailed in section 2.2.1. The assay limit of detection was 11.0 pg/ml. The intra-assay coefficient of variation was less than 12% and the inter-assay coefficient of variation less than 14% at 25 pg/ml. Cortisol concentrations were determined by radio-immunoassay following extraction with diethyl-ether (Brooks and White 1990). The method is described fully in section 2.2.2. The lower limit of detection was 0.27 ng/ml. The intra-assay coefficient of variation was 7% and the inter-assay coefficient of variation 10% at 3.9ng/ml. Median eminence dialysate AVP concentrations were determined in singlicate using an established method (Currie, Gillies et al. 1994) detailed in section 2.2.6. For fractions collected over 30 min epochs during insulin-induced hypoglycaemia 50 μl aliquots were diluted to 100 μl for assay. Samples collected over 2 h longitudinally throughout gestation were assayed undiluted. Intra- and inter-assay coefficients of variation were less than 15% and 16% respectively and the lower limit of detection 1.8 pg/ml. The concentration of CRH in the dialysate was determined in duplicate 100 μl aliquots from

fractions collected overnight prior to insulin challenge using an established assay (Brooks, Power et al. 1989) which is described in section 2.2.7. All of these samples contained measurable quantities of AVP. A single assay run was carried out with intra-assay coefficient of variation of 16% and lower limit of detection of 120 pg/ml.

HPLC separation and fluorimetric assay of amnio acids

The concentration of amino acid neurotransmitters in the dialysate was determined by fluorimetric analysis after separation on a reversed-phase HPLC column. Samples were prederivatised using a modification of a commercially available chemistry package (AccQ.Tag, Millipore, Milford, MA, USA). Briefly, 20 μl sample or standard were mixed with 1 μl 0.5M borax solution in a borosilicate glass tube and 5 μl AccQ.Tag reagent added and vortexed immediately. A 10 μl aliquot of derivatised sample or standard was then loaded onto column. Standards consisted of the amino acid hydrolysate stock supplied with the addition of GABA, homocysteic acid, tryptophan, taurine, histamine, adrenaline, Nor-adrenaline, serotonin, dopamine and L-DOPA. A range of standards were prepared by serial dilution to load 50, 20, 10 and 5 pmol derivatised amino acid onto the column. Since the fluorescent product is then stable, samples and standards could be derivatised some time before loading onto the column and stored at 4C until analysis.

The HPLC system consisted of Waters 625 LC pump and Waters 470 scanning fluorescence detector with excitation and emission wavelengths set at 250 nm and 395 nm respectively. Samples (10 μl) were manually injected. Separation of the derivatised amino acids was achieved at 37C on a reverse phase C18, 4 μm column (Millipore, Milford, MA, USA) with a mobile phase of 0-17% acetonitrile and 100-83% Eluant A at a flow rate of 1ml/min.

Each assay run included 50, 20, 10, and 5 pmol standards along with the samples collected during the insulin challenge for each individual animal. In some cases the identity of the glutamate or GABA peak was confirmed by spiking a further aliquot of the sample with additional amino acid and

demonstrating co-elution of the peak of interest with the additional amino acid. The area under the peak of the fluorescence signal for each amino acid of interest was plotted against concentration and found to be linear over the range 1 pmol to 50 pmol loaded onto the column. The intraassay coefficients of variation at the 10 pmol level for various neurotransmitters were: aspartate 10%, glutamate 8.9%, GABA 9.6%, nor-adrenaline 22.9%, adrenaline 25.9%, and dopamine 12.6%. The interassay coefficients of variation were: aspartate 12.4%, glutamate 10.1%, GABA 11%, nor-adrenaline 25%, adrenaline 28.2%, and dopamine 14.6%.

Statistical analysis

Dialysate and plasma hormone concentrations during the insulin challenge were analysed by analysis of the variance (ANOVA) with repeated measures using the Abacus Concepts, Statview package (version 4.1) for the Apple MacIntosh (Abacus Concepts, Inc., Berkley, CA, USA).

4.4 Results

Detection of AVP, CRF and glutamate in microdialysis samples

Satisfactory probe placement was judged by two means: firstly the detection of AVP in dialysate obtained in the samples collected immediately after surgery, and secondly by inspection of the median eminence and pituitary at euthanasia. The concentration of AVP in dialysate was very variable, presumably reflecting small differences in probe placement, and declined rapidly with time so that by 36 to 48 h after surgery levels were below the assay limit of sensitivity in most animals. We were unable to detect CRH in any samples (even those with high concentrations of AVP). All animals had measurable quantities of AVP at the time of insulin challenge. Several amino acid neurotransmitters were detected in the dialysate (glutamate, GABA, aspartate) but levels were generally low.

Effect of insulin induced hypoglycaemia on release of AVP at the median eminence and plasma ACTH and cortisol concentrations

Because levels of AVP in dialysate varied between animals the data was normalised by expressing AVP concentrations as a percentage of the mean concentration in the two samples prior to insulin challenge. Insulin induced hypoglycaemia produced a significant increase in AVP recovered in the dialysate which was not inhibited by pretreatment with CGP 37849 (ANOVA, Change with time $p<0.02$, Treatment by time interaction $p=0.99$). The AVP response to insulin induced hypoglycaemia is shown in figure 4.1. Plasma ACTH and cortisol concentrations were also normalised for comparison. Insulin challenge elevated plasma ACTH concentrations and this was significantly reduced by pretreatment with CGP 37849 (ANOVA, Change with time $p<0.001$, Treatment by time interaction $p<0.05$). Plasma cortisol concentrations increased significantly with insulin induced hypoglycaemia, but there was no effect of CGP 37849 to blunt the rise in cortisol (ANOVA, Change with time $p<0.01$, Treatment by time interaction $p=0.17$). Levels of glutamate detected in median eminence dialysate samples were low and did not rise following insulin challenge. The plasma ACTH and cortisol responses to insulin induced hypoglycaemia are shown in figure 4.2.

Longitudinal recovery of AVP in dialysate

The time interval from surgery to delivery or fetal demise (in two cases) was quite variable. In total 19 animals were prepared with median eminence dialysis probes and cannulae. Two fetuses died in utero. The time from surgery to labour in the remainder ranged from 2-14 days, with an average duration of 7.1 ± 0.9 days (mean$\pm$SEM). Fetal cortisol concentrations were generally high in the days immediately post surgery. There was a tendency for lower levels in those destined to continue for longer until labour. When daily cortisol concentrations are centred around day of labour there was a significant rise in cortisol levels over the last 2-3 days prior to labour as shown in figure 4.3 (ANOVA; Effect of time $p<0.0001$). Concentrations of AVP in

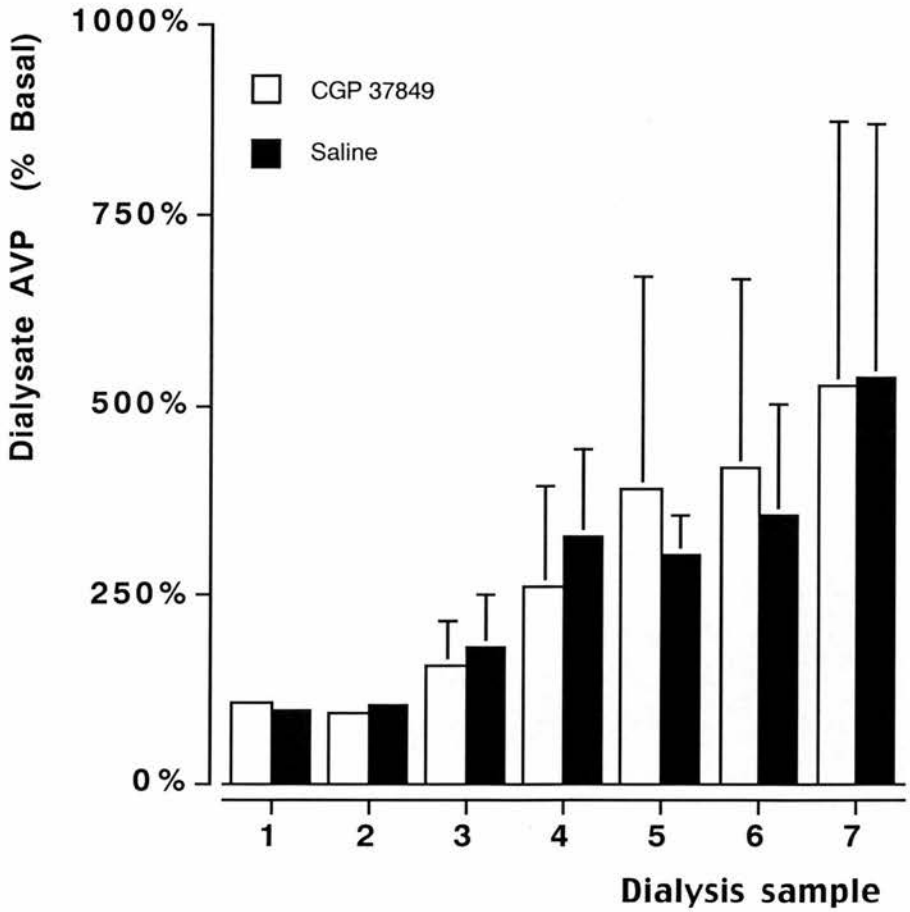
dialysate fell rapidly over the first 24-48 h after surgery to undetectable levels in most animals and remained undetectable even during labour. The AVP recovery in dialysate (as a percentage of initial recovery) with time is shown in figure 4.4. In four fetuses it was possible to sample blood during labour (ANOVA; Effect of time $p>0.05$). In these animals insulin challenge failed to elevate dialysate AVP. The insulin challenge in labour, however, was also without effect on ACTH or cortisol levels. In two fetuses that became significantly hypoxic detectable quantities of AVP were recovered in the dialysate for several days until death in utero as shown in figure 4.5.

Overleaf

Figure 4.1

Effect of insulin-induced hypoglycaemia on AVP concentrations in median eminence dialysate (expressed as a percentage of basal levels). After two basal samples fetuses were pretreated with the NMDA antagonist CGP 37849 (1 mg/kg, iv) (n=5, open bars) or saline vehicle (n=5, filled bars) and were challenged five minutes later with an iv bolus of 10 IU insulin. Values are mean±SEM. Insulin induced hypoglycaemia produced a significant increase in AVP recovered in the dialysate which was not inhibited by pretreatment with CGP 37849 (ANOVA, Change with time $p<0.02$, Treatment by time interaction $p=0.99$).

4.1

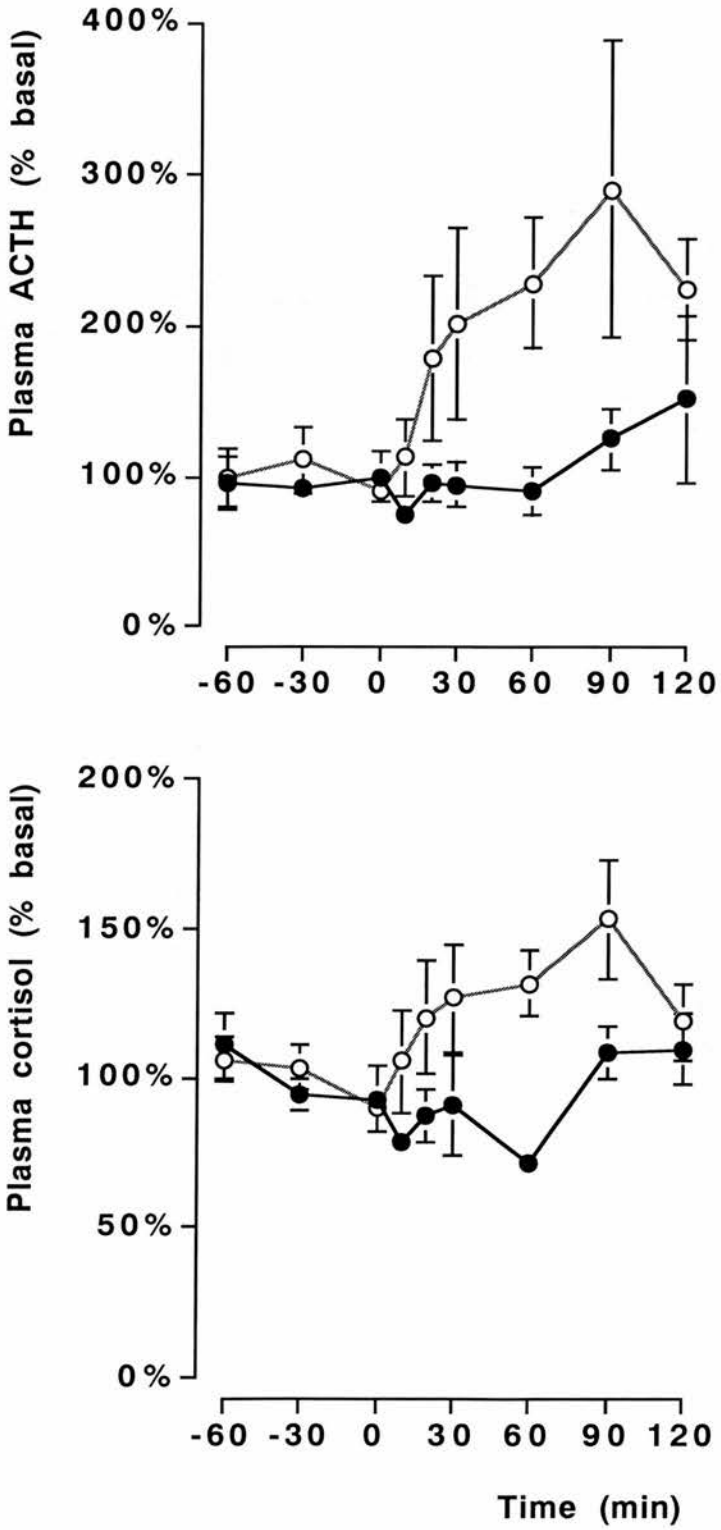

1000%
750%
500%
250%
0%
Dialysate AVP (% Basal)
CGP 37849
Saline
1
2
3
4
5
6
7
Dialysis sample

Overleaf

Figure 4.2

Effect of insulin-induced hypoglycaemia on ACTH (upper panel) and cortisol (lower panel). Fetuses were pretreated with the NMDA antagonist CGP 37849 (n=5, filled circles) or saline vehicle (n=5, open circles) five minutes before challenge with an iv bolus of 10 IU insulin. Values are mean$\pm$SEM. Insulin challenge elevated plasma ACTH concentrations and this was significantly reduced by pretreatment with CGP 37849 (ANOVA, Change with time $p<0.001$, Treatment by time interaction $p<0.05$). Plasma cortisol concentrations increased significantly with insulin-induced hypoglycaemia, but there was no effect of CGP 37849 to blunt the rise in cortisol (ANOVA, Change with time $p<0.01$, Treatment by time interaction $p=0.17$).

4.2

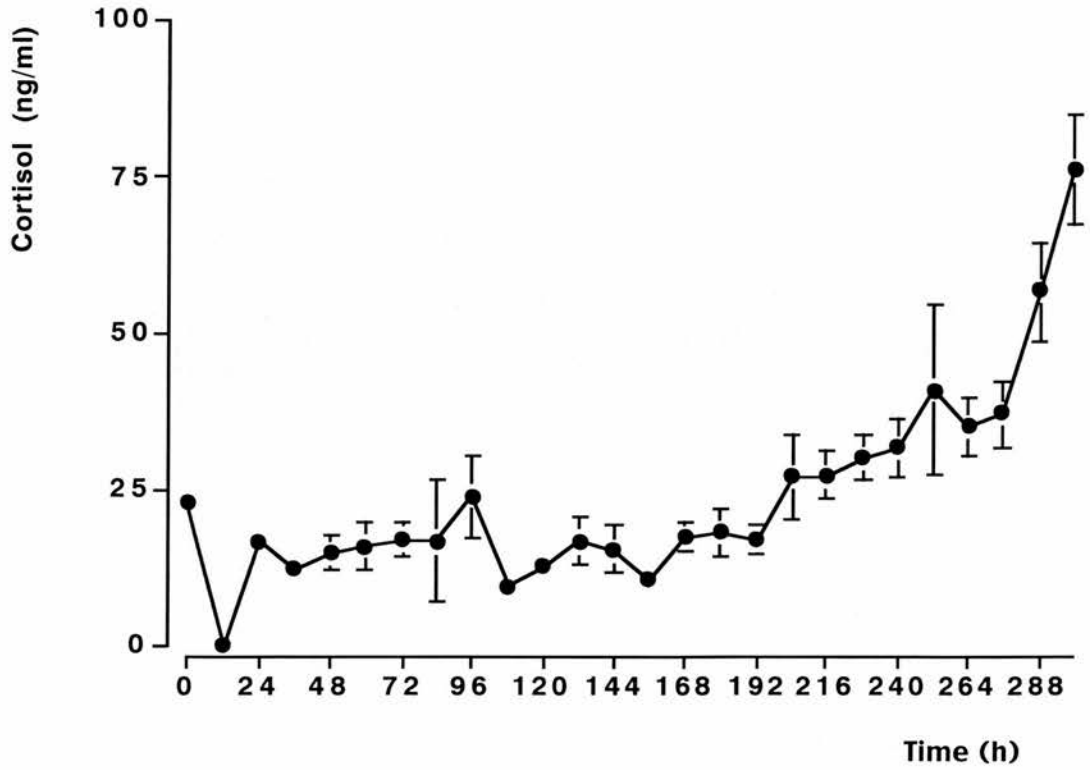

Plasma ACTH (% basal)
400%
300%
200%
100%
0%
-60 -30 0 30 60 90 120
Plasma cortisol (% basal)
200%
150%
100%
50%
0%
-60 -30 0 30 60 90 120
Time (min)

Overleaf

Figure 4.3

Plasma cortisol concentrations centred around the day of delivery. Values are mean$\pm$SEM. Plasma concentrations of cortisol rise significantly prior to labour (ANOVA; Effect of time $p<0.0001$).

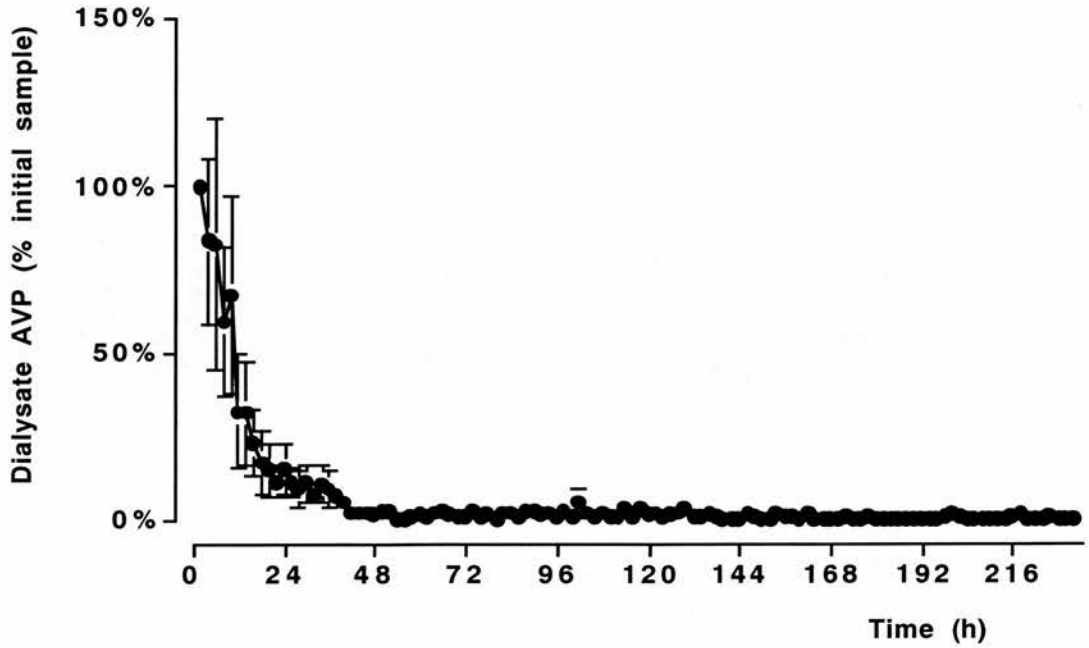
4.3


Cortisol (ng/ml)
100
75
50
25
0
0 24 48 72 96 120 144 168 192 216 240 264 288
Time (h)

Overleaf

Figure 4.4
AVP concentrations in median eminence dialysate (expressed as a percentage of initial recovery) in 10 fetuses followed into labour. Values are mean±SEM.

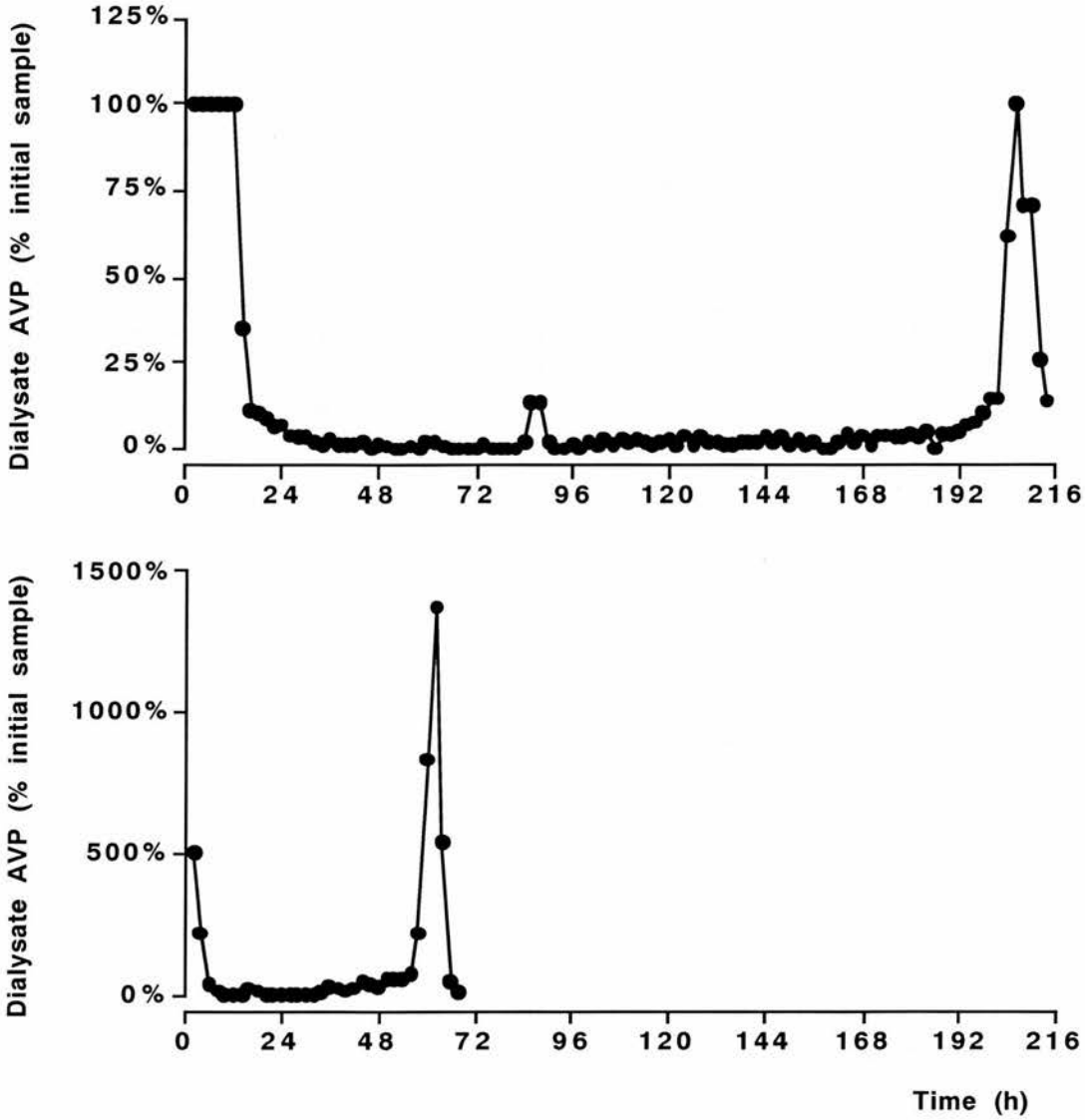
4.4


Dialysate AVP (% initial sample)
150%
100%
50%
0%
0
24
48
72
96
120
144
168
192
216
Time (h)

Overleaf

Figure 4.5
AVP concentrations in median eminence dialysate (expressed as a percentage of initial recovery) in two fetuses which died in utero. Note change of scale on axes.

4.5


Dialysate AVP (% initial sample)
125%
100%
75%
50%
25%
0%
0
24
48
72
96
120
144
168
192
216
Dialysate AVP (% initial sample)
1500%
1000%
500%
0%
0
24
48
72
96
120
144
168
192
216
Time (h)

4.5 Discussion

These results demonstrate that it is possible to use the microdialysis technique in the chronically cannulated ovine fetus to examine neuroendocrine regulation of pituitary hormone secretion. Insulin-induced hypoglycaemia provoked the secretion of AVP at the median eminence and levels increased in parallel with plasma ACTH and cortisol concentrations. Furthermore, pretreatment with CGP 37849 attenuated the ACTH response to insulin induced hypoglycaemia without affecting AVP secretion.

The studies reported in chapter 3 have identified a role for NMDA receptor coupled pathways in the ACTH response to hypoglycaemia and in basal ACTH secretion in late gestation, but were unable to identify if NMDA receptor coupled pathways regulate AVP or CRH secretion. The finding by others that AVP or CRH antagonism does not prevent exogenous NMDA from stimulating ACTH secretion suggests a pituitary site of action (Szeto, Soong et al. 1999). It should be remembered that the distribution of NMDA and CGP 37849 in the brain after intravenous injection may not be the same, and that the finding that exogenous NMDA acts at the pituitary does not necessarily indicate that endogenous excitatory amino acids act principally at the same locus. The role of endogenous transmitters in regulating AVP and CRH secretion are best addressed by studies measuring release at the median eminence

Direct measurement of secretion of releasing factors at the median eminence in the conscious fetus is problematic. The use of a microdialysis system is appealing since, once implanted, the fetus can be replaced in utero and no further access is needed. A number of studies have reported the recovery of AVP and CRH from the brain and median eminence in rodents and larger species using microdialysis (Gabr, Gladfelter et al. 1994; Gabr, Birkle et al. 1995; Levy, Kendrick et al. 1995). When tested in vitro the concentration of AVP recovered in the dialysate using our microdialysis system was about 7% at a flow rate of 2 μl/min. Even with ventriculographically aided placement of the microdialysis probes, however, levels of AVP recovered *in vivo* were quite variable, presumably reflecting

small differences in probe position within the median eminence. Initial levels of AVP recovered in dialysate were high but fell fairly rapidly over 24 to 48 h. Several phenomena may account for this: Firstly, insertion of the probe causes local disruption of nerve fibres and terminals in the median eminence; Secondly, the stress response to the surgery itself; And thirdly, the development of gliosis around the probe tip limits recovery of neurotransmitters from the extracellular fluid space. These considerations limit microdialysis techniques to short term studies unless it is possible to demonstrate similar recovery of neurotransmitter in response to identical challenges over a prolonged period of time (Benveniste and Huttemeier 1990; Stamford 1992; Westerink 1995).

Insulin-induced hypoglycaemia stimulated an increase in plasma concentrations of ACTH and cortisol in the ovine fetus. The same insulin-hypoglycaemia paradigm increases ACTH and cortisol secretion in a number of other species (Redekopp, Irvine et al. 1986; Guillaume, Grino et al. 1989; Ellis, Schmidli et al. 1990; Raff, Papanek et al. 1991; Carnes, Brownfield et al. 1992; Heisler, Tumber et al. 1994). There is good evidence in adult animals that both AVP and CRH are involved in this response. Direct sampling of portal plasma in conscious adult sheep, horses and rats during insulin-induced hypoglycaemia reveals that AVP and CRH are secreted in increased amounts (Redekopp, Irvine et al. 1986; Engler, Pham et al. 1989; Guillaume, Grino et al. 1989; Caraty, Grino et al. 1990), and chronic active immunisation against AVP or CRH diminishes the ACTH response in adult sheep (Guillaume, Conte-Devolx et al. 1992; Guillaume, Conte-Devolx et al. 1992). Insulin induced hypoglycaemia also increases expression of message for AVP and CRH in the rat hypothalamus (Paulmyer-Lacroix, Anglade et al. 1994). In accordance with these observations, we find concentrations of AVP in median eminence dialysate are increased after insulin challenge in the ovine fetus. Unfortunately, CRH was not detectable in any of our samples, but given the low sensitivity of the assay and low recovery of CRH in vitro this is not surprising.

The NMDA receptor antagonist CGP 37849 attenuated the ACTH response to insulin-induced hypoglycaemia in the ovine fetus but did not

affect the release of AVP at the median eminence in response to hypoglycaemia. This strongly suggests that suprapituitary NMDA receptor coupled pathways regulate ACTH secretion through CRH and not AVP. In fact several studies suggest that CRH mediates the action of NMDA to stimulate ACTH secretion. In vitro NMDA stimulates release of CRH from hypothalamic slices (Joanny, Steinberg et al. 1997), and passive immunisation against CRH prevents the ACTH response to NMDA in vivo in the immature rat (Chautard, Boudouresque et al. 1993). Microinjection of glutamate into the amygdala stimulates the secretion of CRH at the median eminence in adult rats (Gabr, Birkle et al. 1995).

The precise site at which intravenously administered NMDA acts to stimulate CRH secretion is not clear. The NMDA receptor is widely distributed throughout the neuroaxis and in theory agonists could act on NMDA receptors found in the pituitary and median eminence (Monyer, Sprengel et al. 1992; Meeker, Greenwood et al. 1994; Bhat, Mahesh et al. 1995), on CRH neuron cell bodies (Aubry, Bartanusz et al. 1996), or at extra-hypothalamic sites (Monyer, Sprengel et al. 1992; Brann and Mahesh 1997). Despite good in vitro recovery of glutamate median eminence dialysate contained little glutamate and concentrations did not change in response to insulin challenge. Our results argue against the median eminence being a major site at which endogenous excitatory amino acids mediate the ACTH response to stress in the ovine fetus. Others, however, have measured changes in median eminence glutamate release in response to restraint stress in rats (Estupina, Abarca et al. 1996).

To assess chronic functioning of the microdialysis system the insulin-induced hypoglycaemia challenge was repeated once the ewe was in labour. Unfortunately, the dose of insulin used did not stimulate ACTH or cortisol secretion in the fetuses of these labouring ewes, perhaps because the high endogenous cortisol concentrations in these fetuses made them relatively insulin resistant. An inhibition of ACTH and cortisol responses to hypoglycaemia has been reported after prior stress (Keller-Wood, Shinsako et al. 1983) or dexamethasone treatment (Carnes, Brownfield et al. 1992). Tantalisingly, in two fetuses very high levels of AVP were recovered in the

dialysate after several days at around the time of fetal demise in utero. This observation indicates that the probes were still capable of recovering AVP after several days.

In summary this study has demonstrated that it is possible to sample AVP secreted at the median eminence in the conscious ovine fetus using a microdialysis technique. Furthermore, it is also shown that AVP secretion in response to insulin induced hypoglycaemia is not regulated by the NMDA receptor. Over the longer term the concentrations of AVP recovered in median eminence dialysate fell to undetectable levels and remained undetectable even in labour. It is not clear if this was a consequence of a decline in the in vivo performance of the probe, perhaps associated with gliosis, or if endogenous secretion of AVP is low in this situation. In two fetuses that died in utero, concentrations of AVP in the dialysate rose to very high levels around the time of death.

5

The role of estrogen in regulating the hypothalamo-pituitary-adrenal axis sensitivity to N-methyl-D-aspartate in the fetal sheep in late gestation

5.1 Abstract

Excitatory amino acid neurotransmitters acting at the NMDA receptor are an important regulator of basal ACTH secretion in late gestation. The ACTH response to NMDA increases with gestation, but the factors responsible for increasing activity of NMDA pathways are unknown. These studies investigate the effect of infusion of 17-β estradiol (100 μg/kg/24 h) or saline vehicle starting on day 120 gestation (term=145) on basal and NMDA stimulated plasma ACTH and cortisol concentrations in the chronically cannulated ovine fetus. Basal plasma ACTH and cortisol concentrations were unchanged over 96 h infusion of 17-β estradiol (n=5) or saline vehicle (n=5). Over this same time period plasma concentrations of PRL were significantly elevated and plasma concentrations of FSH were significantly suppressed by estradiol infusion. After 72 h infusion challenge with AVP (80 ng/kg), CRH (400 μg/kg) and LHRH (125 ng/kg) stimulated an increase in plasma ACTH and cortisol concentrations in both groups, but there was no significant difference between groups. When FSH concentrations were normalised to the preceding baseline, LHRH stimulated a modest increase in plasma FSH concentrations that did not differ between groups. Similarly, after 96 h

infusion intravenous challenge with NMDA (4 mg/kg) stimulated an increase in plasma ACTH and cortisol concentrations, but there was no significant difference between groups. These results indicate that the late gestation increase in HPA axis activity is not dependent upon estrogen.

5.2 Introduction

Excitatory amino acid pathways acting through the NMDA receptor stimulate the ovine fetal HPA axis in late gestation and the ACTH response to exogenous NMDA increases with gestation (Brooks and Howe 1996). The work reported in chapter 3 demonstrated that near term endogenous excitatory amino acids are required for a significant proportion of basal ACTH secretion. Critical to an understanding of the mechanisms that initiate labour is a knowledge of the factors responsible for the increase in the ACTH response to exogenous NMDA are unknown.

In the reproductive axis the effects of agonists at the NMDA receptor on LH secretion are dependent upon the steroid environment (Estienne, Schillo et al. 1990; Pu, Xu et al. 1996). Gonadal steroids modulate both release of transmitter (Carbone, Szwarcfarb et al. 1992; Carbone, Szwarcfarb et al. 1995) and expression of the NMDA receptor (Weiland 1992; Gazzaley, Weiland et al. 1996; Woolley, Weiland et al. 1997), though this has not been substantiated by all researchers (Brann, Zamorano et al. 1993). In the sheep fetus there is some evidence for increasing estrogen concentrations prior to the surge at parturition that could potentially regulate NMDA receptor expression (Findlay and Cox 1970; Nathanielsz, Elsner et al. 1982; Yu, Cabalum et al. 1983). Further support for an increase in biologically active estrogen is drawn from the observation that the levels of gonadotropin in fetal circulation decline over late gestation at around the same time that estrogen negative feedback is developing (Gluckman, Marti-Henneberg et al. 1983).

Reports of the actions of estrogen on the fetal HPA axis have been conflicting. It has demonstration that implants of estradiol increase basal and stimulated ACTH concentrations in the late gestation ovine fetus (Saoud and Wood 1997; Wood and Saoud 1997). In contrast others find direct infusion of

estradiol is without effect (Wang, Matthews et al. 1997). These investigators have not examined HPA axis sensitivity to NMDA.

In the primate fetus it has been proposed that estrogen activates the fetal HPA axis by inducing placental 11βHSD activity (Baggia, Albrecht et al. 1990; Pepe, Waddell et al. 1990). Placental 11βHSD prevents maternal cortisol from reaching the fetal circulation and so releases the fetal pituitary and hypothalamus from inhibition (Pepe, Davies et al. 1994). There are other sites in the HPA axis where feedback can be altered by 11βHSD activity: in adult rats 11βHSD in the brain appears to modulate glucocorticoid negative feedback (Seckl, Dow et al. 1993). It is not clear, however, if brain 11βHSD is estrogen inducible. The ovine placenta, fetal pituitary and hypothalamus also express 11βHSD (Yang 1992).

These experiments test the hypothesis that small increases in fetal estrogen activate the fetal HPA axis by increasing the sensitivity to NMDA.

5.3 Materials and methods

Animals and surgical preparation

Mixed breed sheep with known single insemination dates were used in these experiments. Animals were housed indoors at the MRC field station in mid-Lothian, Scotland. At day 115 gestation (term in our flock is 145 days) fetal sheep were prepared with chronic indwelling jugular, carotid and amniotic cannulae as described in section 2.1. Where there were multiple fetuses only one fetus in each ewe was instrumented. Electrodes (AS 632, Cooner Wire Co, Chatsworth, CA, USA) for recording uterine electromyographic activity were also sutured to the serosal surface of the uterus close to the hysterotomy site. Antibiotics were given to the fetus (10^6 units penicillin, Glaxovet, Uxbridge, UK) and mother (Streptopen, Glaxovet, Uxbridge, UK) for 3 days after surgery. Post-operatively, animals were individually housed in metabolism crates. At least two animals were always housed in the same room to lessen isolation stress on the mother. Animals

were fed twice daily and allowed free access to water. The cannulae were flushed daily with heparinised saline (20 IU/ml) and a small arterial sample withdrawn for blood gas analysis (IL1306; Instrumentation Laboratories, Warrington, Cheshire, UK). Only fetuses with maintained healthy acid-base status (measurements in close agreement with normal ranges of: pH 7.352±0.01; pO2 20.28±0.49 mmHg; pCO2 49.39±0.59 mmHg) were included in the experiment. All experiments were conducted at least 5 days after surgery.

Blood sampling regime

Samples (1 ml unless otherwise stated) were withdrawn from the arterial cannula and replaced with heparinised saline. All samples were collected onto ice and centrifuged within 15 min (3000 rpm, 15 min, 4C), separated and stored frozen at -20C until analysis.

Experimental design

To test the effects of estradiol on the HPA axis fetuses received a continuous infusion of either estradiol or saline vehicle for a period of four days. On the morning of the third day a pituitary challenge of LHRH, AVP and CRH was administered. The estradiol or control infusion was continued and on the morning of the fourth day serial blood samples were withdrawn for a period of four hours to assess pulsatile ACTH and cortisol secretion. At this point animals were challenged with NMDA to estimate the sensitivity of central NMDA receptor coupled pathways.

Infusion and sampling procedure

Blood samples (2 ml) were withdrawn twice daily from fetuses at 0800 h and 2000 h starting on day 119 of gestation. On the morning of day 120 gestation, after the 0800 h blood sample, fetuses commenced an infusion of either 17-β estradiol (Sigma, Poole, UK, iv infusion, 0.5 ml/h) (n=5) or saline

vehicle (n=5). The total dose of estradiol was 100 μg/kg/24 h which has previously been demonstrated to suppress fetal gonadotropin levels at day 110 gestation (Gluckman, Marti-Henneberg et al. 1983). A fetal weight of 3 kg was assumed. After 3 days continuous infusion animals were challenged with a cocktail of AVP (80 ng/kg), CRH (400 μg/kg) and LHRH (125 ng/kg) in 2 ml saline to estimate pituitary sensitivity. Blood samples were taken at -30, -20, -10, 0, 10, 20, 30, 60, 90, and 120 min around the challenge. Fetal haematocrit and blood gases on initial and final blood samples were also analysed. After a further 24 h of estradiol infusion animals underwent a 4 h serial sampling regime with blood samples withdrawn every 10 min. At the end of this time they were challenged intra-arterially with a bolus of NMDA (12 mg/0.6 ml saline) and further blood samples taken at 10, 20, 30, 60, 90, and 120 min after this challenge.

Radioimmunoassay

Immunoreactive $ACTH_{(1-39)}$ was measured in duplicate 100 μl aliquots with a specific 2-site immunoradiometric assay (Brooks and Howe 1996) as described in section 2.2.1, with lower limit of sensitivity of 11 pg/ml and inter and intra-assay coefficients of variation of less than 14% and 12% respectively. Cortisol was determined in duplicate 100 μl aliquots after extraction with diethyl-ether (Brooks and White 1990) as described in section 2.2.2. The inter and intra-assay coefficients of variation were less than 10% and 9% respectively and the lower limit of detection 0.27 ng/ml. The plasma concentrations of FSH and PRL were determined by radioimmunoassay previously validated for sheep (McNeilly and Andrews 1974; McNeilly, Jonassen et al. 1986) and these are detailed fully in sections 2.2.4 and 2.2.5 respectively. The lower limit of detection of the FSH assay was 0.24 ng NIH FSH S14/ml, and the intra-assay coefficient of variation 4.9%. The corresponding values for the prolactin assay were 0.54 ng NIH PRL S15/ml,

inter and intra-assay coefficients of variation were less than 15% and 14% respectively.

Statistical analysis

Hormone concentrations (ACTH, cortisol, PRL and FSH) in the twice daily samples from control and treated fetuses were compared by two factor analysis of the variance with post hoc analysis of individual means by Scheffe's F test (Statview 4.1, Abacus Concepts, Berkeley, on Apple Macintosh computer system). Plasma ACTH and cortisol concentrations in control and treated fetuses in response to pituitary challenge on day 3 of the estradiol infusion were similarly compared by two factor analysis of the variance. Since estradiol was found to significantly suppress basal FSH secretion, the pituitary FSH responses to LHRH were analysed after normalising the amplitude of the FSH response to the basal FSH. Individual plasma ACTH, cortisol and FSH profiles during the 4 h serial sampling period on the fourth day were analysed using a computer pulse detection program (Munro, Zaristow software, Haddington, Scotland). Briefly, the program generates a baseline by examining local nadirs, and then detects pulses as increments of two standard deviations or more from this baseline. Plasma ACTH, cortisol and FSH levels in control and treated fetuses during the 4 h sampling period and after NMDA challenge were then compared by two factor ANOVA.

5.4 Results

Effect of estradiol infusion on basal and NMDA stimulated ACTH and cortisol concentrations, and on pituitary sensitivity to AVP and CRH.

The plasma concentrations of ACTH and cortisol in the daily samples over the course of the experiment are shown in figure 5.1. Basal ACTH and cortisol concentrations were similar in the two groups prior to and during estradiol infusion (ANOVA, Main effect for treatment for ACTH $p=0.67$ and for

cortisol p=0.49). Plasma ACTH and cortisol concentrations were elevated on the evening after the morning pituitary challenge on day 4, but there was no significant difference between groups (ANOVA, treatment by time interaction for ACTH, p=0.98; and for cortisol, p=0.98).

Pituitary and adrenal responses to AVP and CRH challenge on day 4 (after 72 h infusion) are shown in figure 5.2. Plasma ACTH concentrations peaked 10 min after challenge with AVP and CRH and declined thereafter, but were still elevated above basal at 120 min (ANOVA, Effect of time $p<0.001$). There were no significant differences between estradiol and saline infused groups (ANOVA, Treatment by time interaction p=0.97). Similarly, plasma cortisol concentrations peaked at 20 min, but remained elevated through to 120 min (ANOVA, Effect of time $p<0.001$). There were no significant differences between the groups (ANOVA, Treatment by time interaction, p=0.92).

The basal plasma ACTH and cortisol concentrations and the changes in response to NMDA challenge on day 5 (after 96 h infusion) are shown in figure 5.3. There were no significant differences in ACTH or cortisol concentrations between control and estrogen treated animals. Plasma ACTH concentrations peaked at 30 min after NMDA challenge and remained elevated at 120 min, but there were no significant differences between estradiol and saline infused animals (ANOVA, Treatment by time interaction, p=0.99). Cortisol concentrations also rose rapidly after NMDA challenge and remained elevated, and were unaffected by estradiol infusion (ANOVA, Treatment by time interaction, p=0.95). When individual hormone profiles were subjected to pulse analysis no pulses were detected.

Effect of estradiol infusion on basal and NMDA stimulated FSH concentrations, and on pituitary sensitivity to LHRH

The plasma concentrations of FSH in the daily samples over the course of the experiment are shown in figure 5.4. Basal FSH concentrations declined significantly in the estradiol infused animals, and were below the

assay limit of sensitivity after 48 h (ANOVA, Treatment by time interaction $p<0.01$).

The pituitary response to LHRH challenge on day 4 (after 72 h infusion) is shown in figure 5.5. Plasma FSH concentrations were significantly higher in the saline infused group (ANOVA, Main effect for treatment $p<0.05$). When plasma FSH concentrations are normalised by expressing them as a percentage of baseline values, there are no significant differences in the pituitary FSH response to LHRH (ANOVA, Treatment by time interaction, $p=0.45$).

The basal plasma FSH concentrations and the changes in response to NMDA challenge on day 5 (after 96 h infusion) are shown in figure 5.6. Plasma FSH concentrations were lower in the estradiol infused group and there was an increase in FSH concentrations over the duration of the sampling period (ANOVA, Effect of time $p<0.01$, Treatment by time interaction $p=0.3$). When FSH concentrations after NMDA challenge are normalised to the 30 min preceding challenge, then neither group is seen to respond to NMDA challenge (ANOVA, Treatment by time interaction $p=0.6$).

Effect of estradiol on PRL concentrations

Daily plasma PRL concentrations during the infusion are shown in figure 5.4. Estradiol infusion resulted in a significant increase in PRL concentrations over the duration of the experiment when expressed as a percentage increase over basal (pre-infusion) levels wheras PRL concentrations in the saline infused group were unchanged (ANOVA, Effect of time $p<0.001$, Treatment by time interaction p,0.001).

Uterine activity

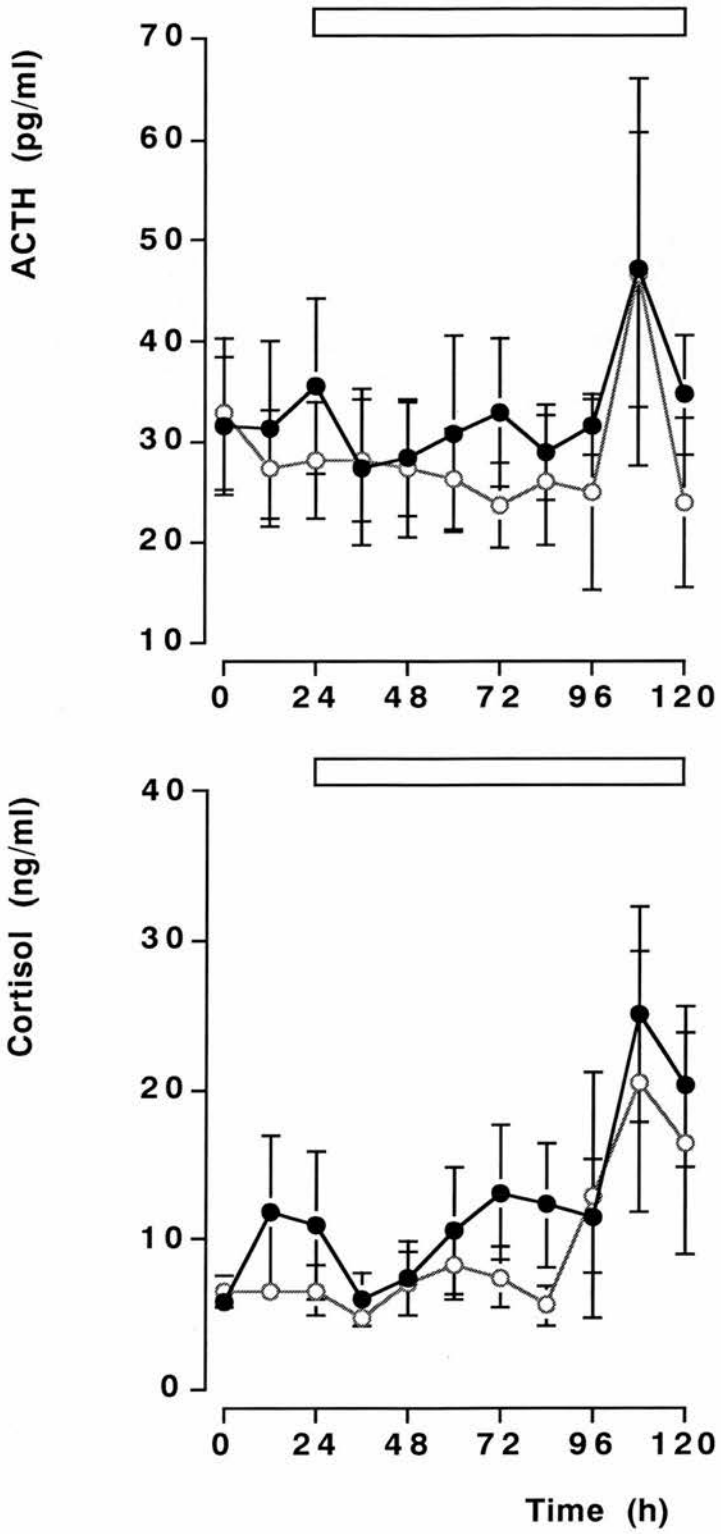
Throughout the experiment all animals demonstrated a pattern of uterine activity consistent with contractures (Nathanielsz, Bailey et al. 1980; Farber, Guissani et al. 1997), indicating that this dose of estradiol was not sufficient to precipitate labour.

Overleaf

Figure 5.1

Plasma ACTH (upper panel) and cortisol (lower panel) concentrations for the day before and during four days intravenous infusion (indicated by the open bar) of estradiol (n=5, filled circles) or saline vehicle (n=5, open circles). Values are mean$\pm$SEM. Estradiol infusion had no effect on basal plasma ACTH or cortisol concentrations (ANOVA, Treatment by time interaction for ACTH p=0.98 and for cortisol p=0.98).

5.1

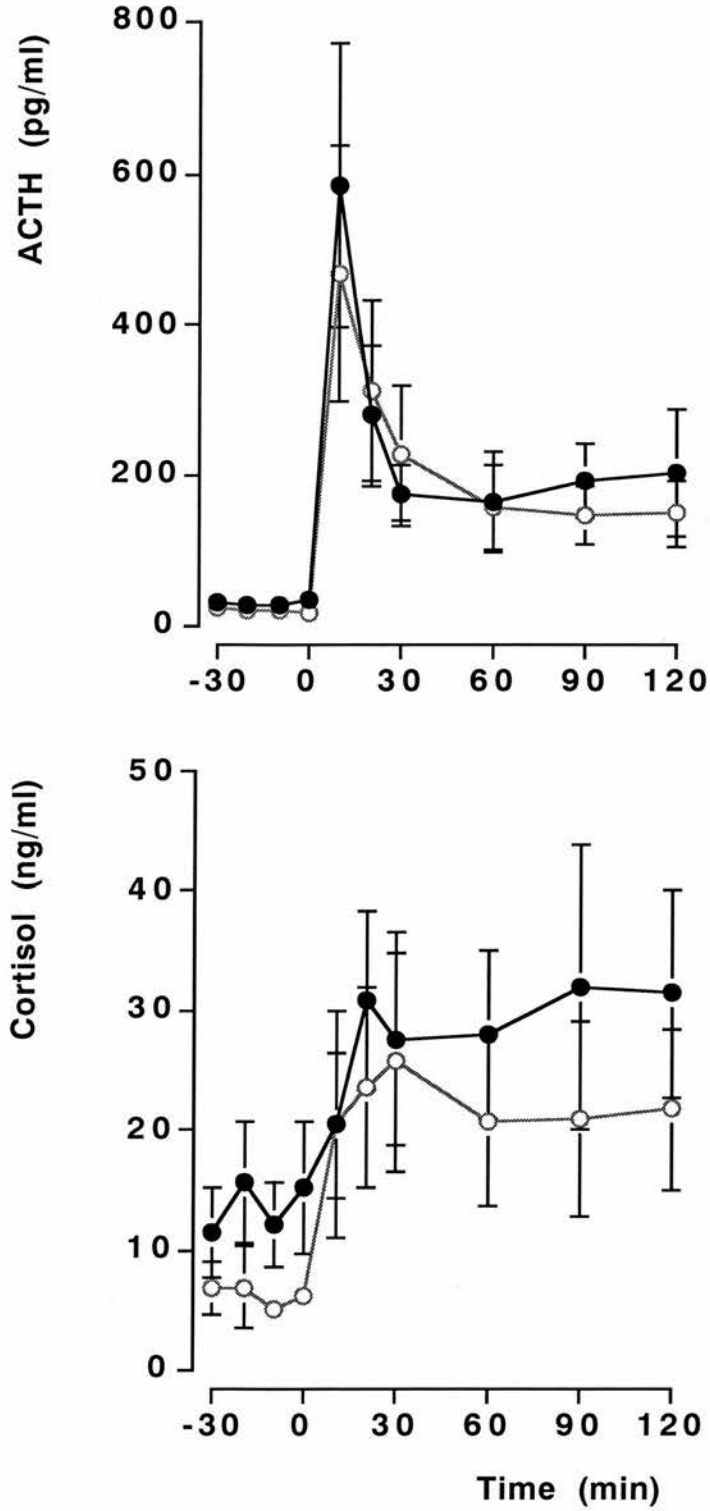

ACTH (pg/ml)
70
60
50
40
30
20
10
0
24
48
72
96
120
Cortisol (ng/ml)
40
30
20
10
0
0
24
48
72
96
120
Time (h)

Overleaf

Figure 5.2

Plasma ACTH (upper panel) and cortisol (lower panel) concentrations following intravenous challenge with CRH (400 μg/kg) and AVP (80 ng/kg) in estradiol (n=5, filled circles) or saline vehicle (n=5, open circles) infused fetuses. Values are mean±SEM. The pituitary responses did not differ between groups (ANOVA, Treatment by time interaction for ACTH p=0.97, and for cortisol p=0.92).

5.2

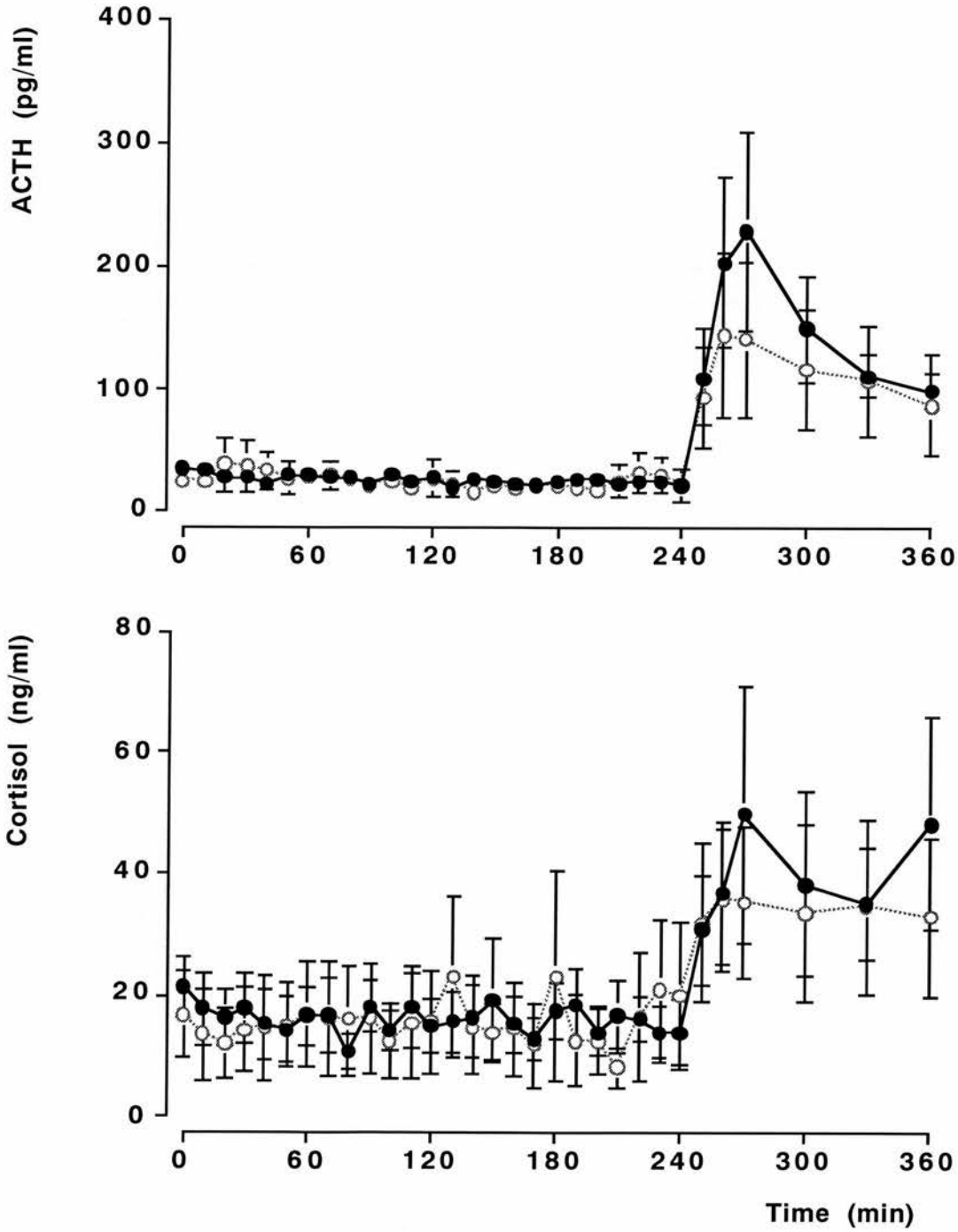

ACTH (pg/ml)
800
600
400
200
0
-30
0
30
60
90
120
Cortisol (ng/ml)
50
40
30
20
10
0
-30
0
30
60
90
120
Time (min)

Overleaf

Figure 5.3

Plasma ACTH (upper panel) and cortisol (lower panel) concentrations in ten min serial samples collected over four hours after four days of estradiol infusion (n=5, filled circles) or saline vehicle (n=5, open circles). A bolus of NMDA (4 mg/kg intra-arterial) was given after four hours and samples collected over a further two hours. Values are mean±SEM. Estradiol had no significant effect on either basal or NMDA stimulated ACTH or cortisol concentrations (ANOVA, Treatment by time interaction p=0.99 for ACTH and p=0.95 for cortisol).

5.3

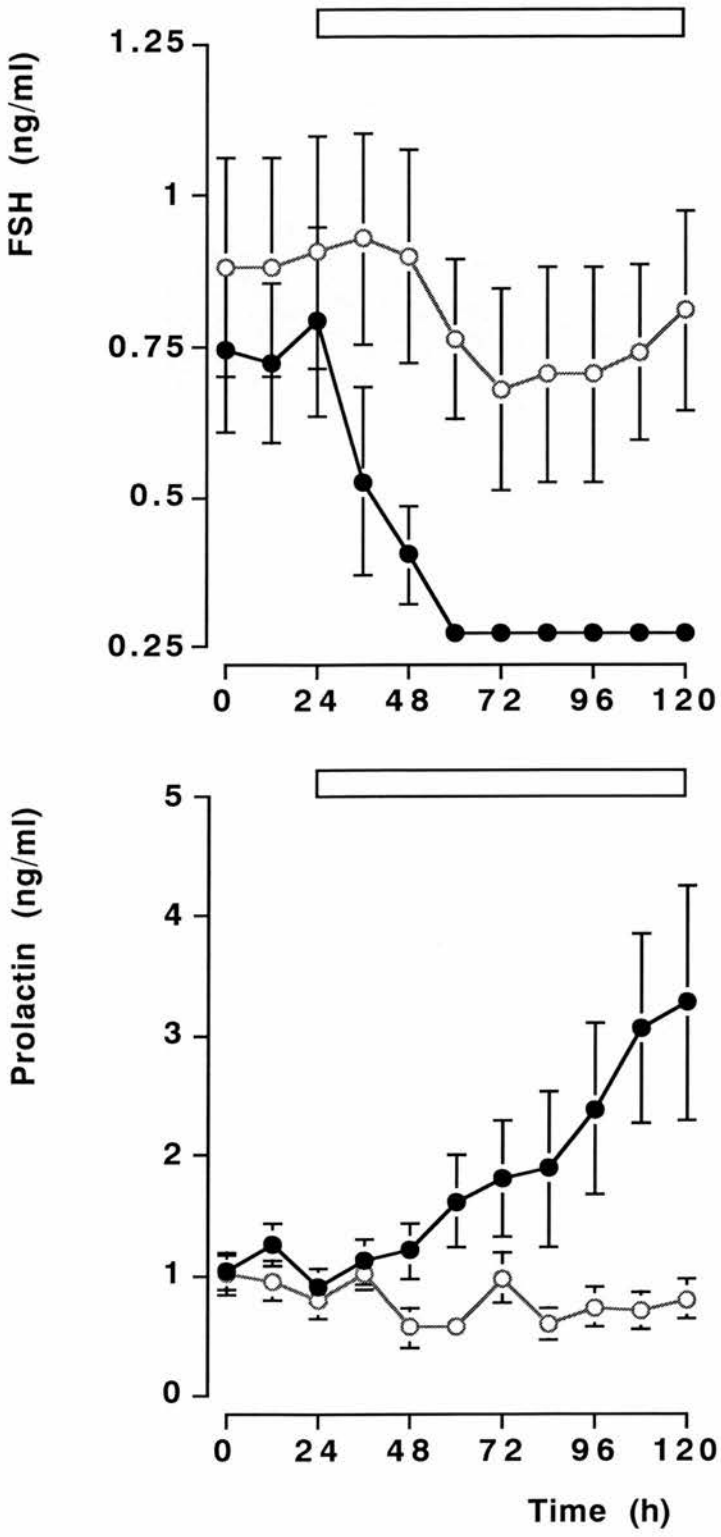

ACTH (pg/ml)
400
300
200
100
0
0
60
120
180
240
300
360
Cortisol (ng/ml)
80
60
40
20
0
0
60
120
180
240
300
360
Time (min)

Overleaf

Figure 5.4

Plasma FSH (upper panel) and PRL (lower panel) concentrations for the day before and during four days intravenous infusion (shown by the open bar) of estradiol (n=5, filled circles) or saline vehicle (n=5, open circles). Values are mean$\pm$SEM. Estradiol infusion resulted in a significant fall in plasma FSH concentrations and an increase in plasma PRL concentrations over the course of the infusion (ANOVA, Treatment by time interaction for FSH $p<0.01$ and for PRL $p<0.001$).

5.4

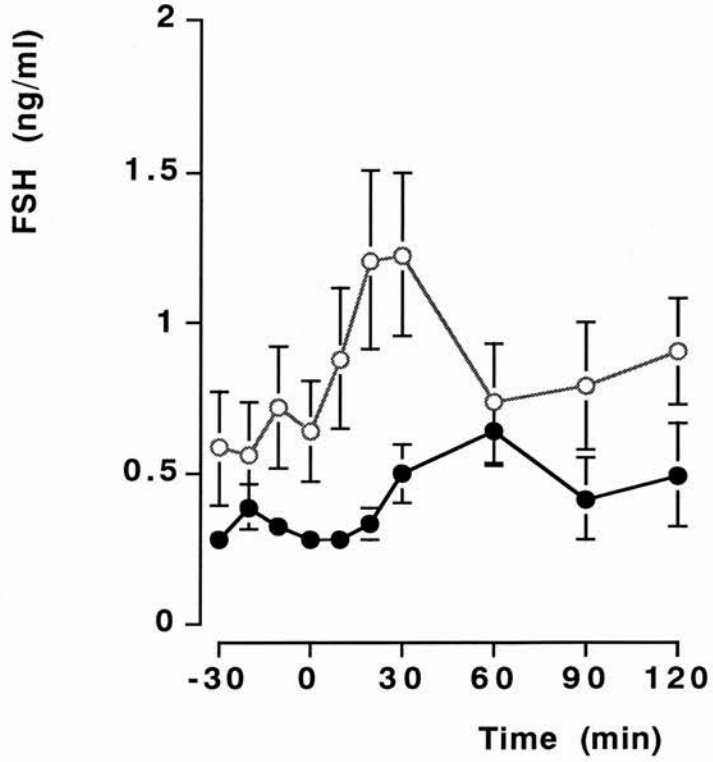

FSH (ng/ml)
1.25
1
0.75
0.5
0.25
0
24
48
72
96
120
Prolactin (ng/ml)
5
4
3
2
1
0
Time (h)

Overleaf

Figure 5.5

Plasma FSH concentrations following intravenous challenge with LHRH (125ng/kg) in estradiol (n=5, filled circles) or saline vehicle (n=5, open circles) infused fetuses. Values are mean$\pm$SEM. FSH concentrations were significantly higher in the saline infused group (ANOVA, Effect of treatment $p<0.05$). After normalisation of FSH concentrations to the preceding baseline, there were no significant differences between groups in response to LHRH (ANOVA, Treatment by time interaction $p=0.45$).

5.5

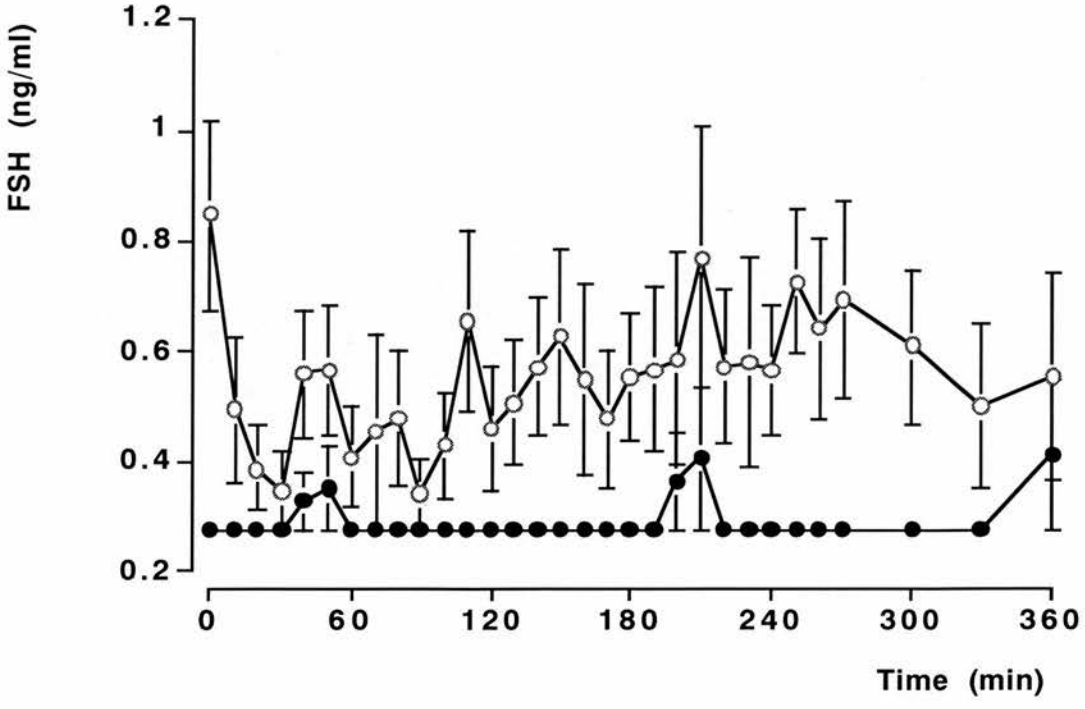


Overleaf

Figure 5.6

Plasma FSH concentrations in ten minute serial samples collected over four hours after four days of estradiol infusion (n=5, filled circles) or saline vehicle (n=5, open circles). A bolus of NMDA (4 mg/kg intra-arterial) was given after four h and samples collected over a further two h. Values are mean$\pm$SEM. FSH concentrations were significantly higher in the saline infused group (ANOVA, Main effect of treatment $p<0/05$). When FSH values were normalised to the preceding baseline, NMDA challenge did not stimulate an increase in FSH (ANOVA, Treatment by time interaction $p=0.6$).

5.6



5.5 Discussion

These results demonstrate physiological levels of estradiol are without effect on the ovine fetal HPA axis at day 120 gestation. Four days continuous estradiol infusion had no effect on basal ACTH or cortisol concentrations, and did not alter pituitary sensitivity to AVP and CRH. Furthermore, the sensitivity of the HPA axis to challenge with NMDA was unchanged. In contrast, there was a significant suppression of the already low FSH levels over the four days of the infusion indicating the hypothalamo-pituitary-gonadal (HPG) axis to be sensitive to estradiol. This action of estradiol appears to occur at a supra-pituitary level since the amplitude of the pituitary FSH response to LHRH challenge was unchanged by estradiol infusion. Estradiol also had an action to elevate daily PRL concentrations.

Estradiol infusion from day 120 gestation in the ovine fetus is seen to affect the HPG axis and PRL secretion, but not the HPA axis, suggesting that estradiol is not responsible for the preparturient increase in HPA activity in this species. Similar findings are reported using an infusion of 100 μg/24 h, but with different stress tests of HPA activity (Wang, Matthews et al. 1997). Others, however, report that in fetal sheep at a similar gestation subcutaneous implants releasing 0.24 mg estradiol per day result in increased basal ACTH concentrations after 5 days (Saoud and Wood 1997; Wood and Saoud 1997). In this study a total dose of 1.2 mg was administered over a 96 h period without effect. This dose and route of estradiol administration has previously been demonstrated to produce biological effects despite there being only a modest elevation of conjugated estradiol with no increase in levels of free estradiol (Gluckman, Marti-Henneberg et al. 1983). The concentrations of estradiol achieved by this infusion are comparable to the endogenous concentrations seen in later gestation. At day 120 gestation endogenous unconjugated estradiol levels are low (Nathanielsz, Elsner et al. 1982; Yu, Cabalum et al. 1983). Estradiol production does, however, increase from mid to late gestation as evidenced by the rise in circulating conjugated estradiol (Findlay and Cox 1970; Carnegie and Robertson 1978) and by the decline in FSH concentrations

(Gluckman, Marti-Henneberg et al. 1983). The current study indicates that modest elevations of estradiol, within the physiological range, at a gestation shortly before the concentrations of ACTH and cortisol in the fetal circulation start to rise, are without effect on the ovine fetal HPA axis.

This study has also examined the effects of estradiol on basal activity in the HPG axis and on the response to challenge with NMDA. Levels of gonadotropins in the fetal ovine circulation are highest at mid-gestation and decline with advancing gestation (Foster, Roach et al. 1972; Sklar, Mueller et al. 1981). Negative feedback actions of estradiol develop somewhere between 90 and 105 days gestation and at least in part cause the decline in gonadotropins (Gluckman, Marti-Henneberg et al. 1983). In accordance with this we find that estradiol infusion suppresses basal FSH concentrations but not the pituitary FSH response to LHRH challenge. Stimulation of the HPG axis with NMDA did not elevate FSH secretion in control animals. In contrast, the same dose of NMDA is known to stimulate LH secretion at this gestation (Brooks and Howe 1996). The lack of FSH response to NMDA may be related to low pituitary FSH responsiveness to LHRH. Challenge with LHRH produced an approximate doubling of FSH concentrations in our control animals, wheras, at the same gestation, the same dose of LHRH is reported to increase LH by 10 to 20 fold (Brooks and Howe 1996).

During late gestation the fetal HPA axis becomes more sensitive to NMDA challenge (Brooks and Howe 1996) and the hypothesis was advanced that increasing responsiveness to NMDA was estrogen dependent. The absence of effect of estradiol on the HPA axis despite suppression of FSH and stimulation of PRL suggests that the mechanisms allowing sustained activation of the HPA axis in the late gestation fetal sheep do not depend upon estrogen. It is possible, however, that at day 120 the fetal sheep is developmentally too immature to show estradiol enhancement of the ACTH response to NMDA: in ovariectomised rats, the stimulatory effect of estrogen on the LH response to NMDA is linked to development events at puberty, since before this time treatment with estrogen and progesterone suppresses LH (Carbone, Szwarcfarb et al. 1995).

In summary, this study does not support the concept that estradiol increases NMDA receptor stimulated ACTH release in the maturing ovine fetus. There may, though be a role for other steroids in the increase in HPA axis responsiveness to NMDA. Infusion of cortisol in late gestation has a positive action on the ovine fetal HPA axis (Apostolakis, Longo et al. 1994) and chronic corticosterone treatment in adult rats has been shown to increase hippocampal expression of NMDA receptor message (Weiland, Orchinik et al. 1997).

6

Evidence that central Neuropeptide-Y pathways regulate hypothalamo-pituitary-adrenal axis activity in the late gestation fetal sheep

6.1 Abstract

The HPA axis in adult animals has a role in regulating metabolism. Central NPY containing neurons in the arcuate nucleus are important in signalling the metabolic state to the HPA axis. The potential role of NPY in the regulation of pituitary-adrenal function was examined in fetal sheep at day 125 gestation. Fetuses were prepared with lateral cerebral ventricle cannulae and NPY(6.5 μg, or 30 μg in 50 μl saline) or saline vehicle administered on consecutive days as a bolus over 5 min. A significant increase in plasma concentrations of ACTH but not cortisol occurred in response to the highest dose of NPY.

6.2 Introduction

In adult animals the HPA axis participates in the endocrine responses during the transition from the fed to the fasted state, and during refeeding (Dallman, Strack et al. 1993). Similar functions can be envisaged during fetal life, and it is even possible that the late gestation increase in ACTH and cortisol secretion reflects a kind of intra-uterine starvation from which the fetus escapes by initiating labour. The hypothalamic pathways regulating energy balance include an NPY projection from the arcuate nucleus to the

PVN that appears to be present in the late gestation fetus (Warnes, Morris et al. 1998), though it has not been established that this pathway is functional.

Neuropeptide-Y is stimulatory action on the HPA axis. Administration of NPY either directly into the PVN or into the cerebral ventricles results in elevated plasma concentrations of ACTH or cortisol in the rat and dog (Harfstrand, Eneroth et al. 1987; Wahlestedt, Skagerberg et al. 1987; Inoue, Inui et al. 1989). Furthermore, administration of specific antibodies to NPY via the third cerebral ventricle significantly inhibits the ACTH and cortisol response to insulin-induced hypoglycaemia (Inui, Inoue et al. 1990). Thus, endogenous NPY appears to be involved in the regulation of pituitary adrenal function. Further evidence for a functional role for NPY in the control of pituitary adrenal function is provided by studies which demonstrate a decrease in the expression of NPY mRNA in the arcuate nucleus (from where afferent fibres project to the PVN) after adrenalectomy (Bai, Yamano et al. 1985). The effect of adrenalectomy on NPY expression is reversed by glucocorticoid treatment (White, Dean et al. 1990). In addition, a diurnal rhythm of NPY immunoreactivity has been detected in the suprachiasmatic and arcuate nuclei as well as the parvocellular region of the PVN (Jhanwar-Uniyal, Beck et al. 1990).

To date there is no information concerning the role of central NPY pathways in the control of pituitary-adrenal function during fetal life. It is known that the NPY gene is expressed in the fetal sheep mediobasal hypothalamus from at least day 110 gestation, and expression increases with gestation and in response to maternal undernutrition (Warnes, Morris et al. 1998). In this study the response of the ovine fetal HPA axis to intracerebroventricularly administered NPY is examined.

6.3 Materials and methods

Animals and surgical preparation

Mixed breed sheep with known single insemination dates were used in these experiments. Between day 115-120 gestation (term approximately 145

days) four fetal sheep were prepared with chronic indwelling jugular, carotid and amniotic cannulae as previously described in section 2.1. The lateral cerebral ventricle was catheterised using previously described methods (Brooks 1992). Briefly, a 2 mm burr hole was made in the parietal bone 4-5 mm lateral to the sagittal suture and 5 mm rostral to the coronal suture. A holding plate manufactured from a Vacutainer needle holder (Beckton-Dickinson Vacutainer Systems) was anchored over the hole using stainless steel screws and dental acrylic. A 5 mm guide cannula (modified 20 G Vacutainer needle) was then screwed into the holding plate. A 26 G needle attached to a 20 cm length of polyethylene tubing (PE20, Clay Adams, NJ, USA) filled with sterile saline, was then lowered until the saline entered the lateral ventricle by gravity flow. The needle was then secured at the correct depth with dental acrylic and a 150 cm polyethylene cannula (PE20) was attached and exteriorised through the ewes flank along with the vascular catheters. The dead volume of the lateral ventricle cannula was 100 μl. Where there were multiple fetuses only one fetus in each sheep was cannulated.

Within 24 h of surgery animals were individually housed in metabolism crates and maintained for the duration of the experiment under a constant 12:12 h light dark cycle. The cannulae were flushed daily with heparinised saline (20 IU/ml) and a small arterial sample withdrawn for blood gas analysis (IL1306; Instrumentation Laboratories, Warrington, Cheshire, UK). Only fetuses with maintained healthy acid-base status (measurements in close agreement with normal ranges of: pH 7.352±0.01; pO_2 20.28±0.49 mmHg; pCO_2 49.39±0.59 mmHg) were included in the experiment. Antibiotics were given to the fetus (10^6 units penicillin; Crystapen, Glaxovet) and mother (Streptopen, Glaxovet) for 3 days after surgery. All experiments were conducted at least 5 days after surgery.

Blood sampling regime

Samples (1 ml) were withdrawn from the arterial cannula and replaced with heparinised saline. All samples were collected onto ice and centrifuged

within 15 min (3000 rpm, 15 min, 4C), separated and stored frozen at -20C until analysis.

Experiment : Effect of intracerebroventricular NPY on ACTH secretion

Between day 120-122 gestation beginning at 0800 h, fetuses received an intracerebroventricular (icv) injection over 5 min of saline, 6.5 μg and 30 μg human NPY in a volume of 50 μl on each of three consecutive days. Peptides were purchased from Cambridge Research Biochemicals (Northwich, Cheshire, UK) and dissolved freshly each day in sterile saline without heparin. Blood samples (1 ml) were withdrawn at -30, -15, and 0 min before and 15, 30, 60, 90, 120, 150 and 180 min after the injection.

Hormone determination.

Samples were assayed in duplicate for immunoreactive ACTH $_{(1\text{-}39)}$ by a specific 2-site immunoradiometric assay (Brooks and Howe 1996) which is described in section 2.2.1. The assay limit of detection was 11.0 pg/ml. Inter- and intra-assay coefficients of variation were less than 14% and 12% respectively. Cortisol concentrations were determined by radio-immunoassay following extraction with diethyl-ether (Brooks and White 1990) described in section 2.2.2. The lower limit of detection was 0.27 ng/ml. Inter- and intra-assay coefficients of variation were less than 10% and 9%, respectively.

Statistical analysis.

Basal ACTH concentrations in the blood samples taken before the administration of NPY were averaged and subtracted from all subsequent samples. The influence of saline or NPY on plasma ACTH concentrations was then compared by two factor analysis of the variance (ANOVA) with repeated measures using the Abacus Concepts, Statview package (version 4.1) for the Apple MacIntosh (Abacus Concepts, Inc., Berkley, CA, USA).

6.4 Results

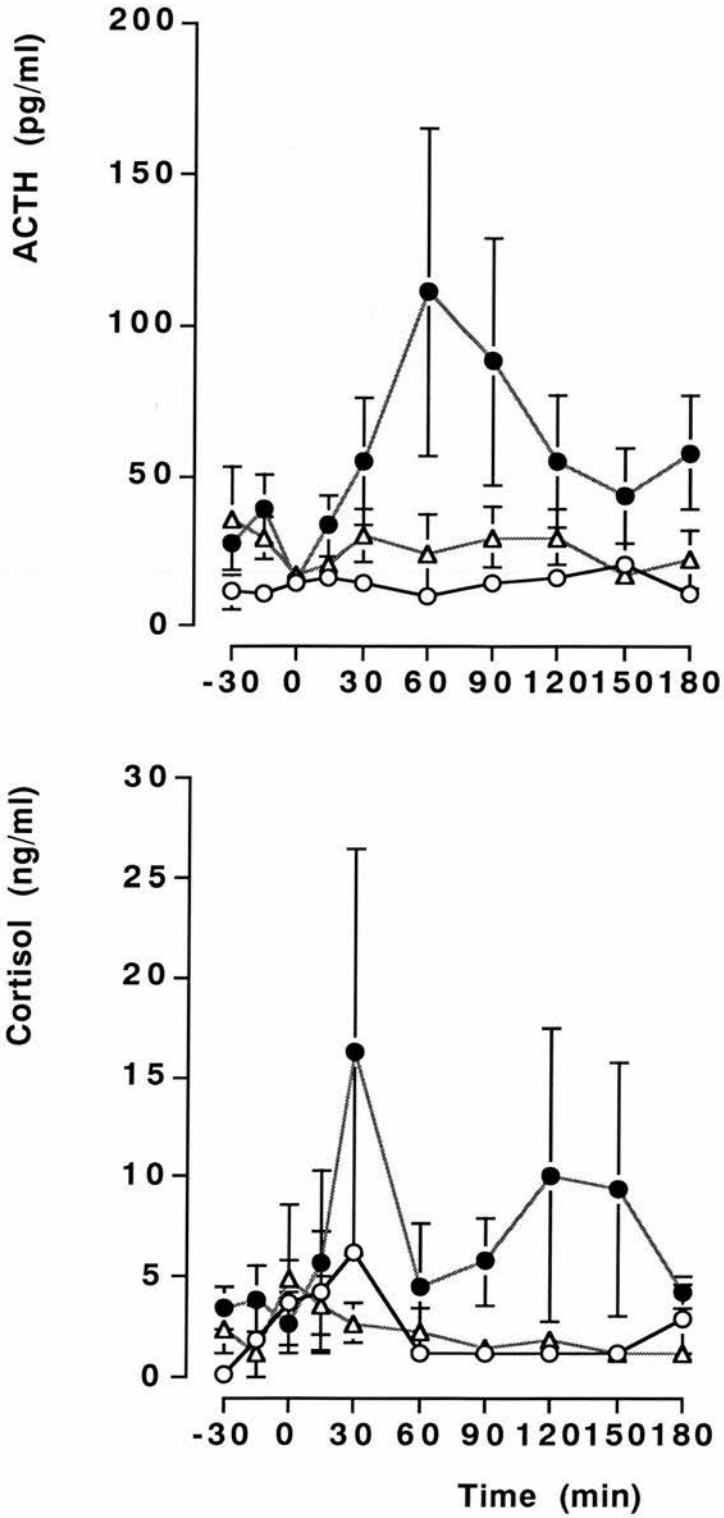
Intracerebroventricular injection of a bolus of NPY stimulated a modest increase in plasma ACTH which peaked at 60 min post injection. There was a significant treatment by time interaction ($p<0.03$). The difference between the high dose (30 μg) and saline groups with respect to time was significant ($p<0.03$), but there were no significant difference between either saline and low dose (6.5 μg) groups or between low and high dose groups. Plasma cortisol concentrations did not differ between the groups.

Overleaf

Figure 6.1

Plasma ACTH (upper panel) and cortisol (lower panel) concentrations in response to icv NPY. On consecutive days starting at day 120 gestation, saline vehicle (open circles), 6.5 μg NPY (triangles) and 30 μg NPY (filled circles) were administered into the lateral cerebral ventricles of fetal sheep (n=4). Values are mean±SEM. Treatment with 30 μg NPY stimulated a significant increase in plasma ACTH concentrations (ANOVA; Treatment by time interaction $p<0.03$). The ACTH response to the lower dose of NPY failed to reach significance, as did the cortisol responses.

6.1


ACTH (pg/ml)
200
150
100
50
0
-30 0 30 60 90 120 150 180
Cortisol (ng/ml)
30
25
20
15
10
5
0
-30 0 30 60 90 120 150 180
Time (min)

6.5 Discussion

This study demonstrates that central administration of NPY in fetal sheep at around day 120 gestation increases plasma concentrations of ACTH, confirming a role for central NPY pathways in regulating ACTH secretion. We were unable, however, to detect any changes in plasma cortisol concentrations.

Central NPY pathways have been implicated in the regulation of ACTH secretion in other species. For instance, intracerebroventricular injection of NPY in the rat and dog results in increased plasma ACTH concentrations as does direct injection of NPY into the PVN of anaesthetized rats (Harfstrand, Eneroth et al. 1987; Wahlestedt, Skagerberg et al. 1987; Inoue, Inui et al. 1989). Furthermore, recent studies have demonstrated that in adult sheep, intracerebroventricular administration of NPY stimulates an increase in plasma ACTH and cortisol concentrations (Porter, Naylor et al. 1993; Brooks, Howe et al. 1994). Part of the effect of NPY on HPA activity is likely to be mediated via the secretion of CRH from the hypothalamus since NPY has been shown to increase the concentrations of CRH in the median eminence in association with increased plasma concentrations of ACTH (Haas and george 1987). Additionally, NPY stimulates the secretion of CRH from rat hypothalami incubated *in vitro* in a dose dependent manner (Tsagarakis, Rees et al. 1989). The most convincing evidence that endogenous NPY contributes to the physiological regulation of ACTH secretion is provided by a report which shows that central immunoneutralization of NPY using a specific antibody to NPY substantially blocks the increase in ACTH following insulin-induced hypoglycaemia (Inui, Inoue et al. 1990). In adult sheep administration of a bolus of 6.5 μg NPY into the third cerebral ventricle stimulates an increase in plasma ACTH concentrations (Brooks, Howe et al. 1994). It is not clear if the difference in sensitivity between adult and fetal sheep reflects a maturational change, or simply differences in route of administration (third versus lateral cerebral ventricle). None-the-less, these results do demonstrate that central NPY pathways are coupled to ACTH secretion in the ovine fetus by at least day 125 gestation.

The lack of significant cortisol response to elevated plasma ACTH secretions may be due to the relatively low adrenal sensitivity at this time (Rose, Meis et al. 1982). An alternative explanation is that much of the measured increase in ACTH activity is in fact larger molecular weight precursor with low biological activity. Others have demonstrated a developmental change in the ratio of low to high molecular weight ACTH species, with increasing amounts of bioactive ACTH secreted with advancing gestation (Brieu and Durand 1987; Saphier, Glynn et al. 1993; Carr, Jacobs et al. 1995).

In adult animals central NPY pathways are part of a complex system regulating behavioural and metabolic responses to nutritional state (Schwartz and Seeley 1997; Schwartz 1998; Woldbye and Larsen 1998). Chronic administration of NPY into the hypothalamus leads to obesity (Stanley, Kyrkouli et al. 1986; Zarjevski, Cusin et al. 1993). There is considerable redundancy in the system, though, as body mass and daily food intake are not significantly disturbed in mice lacking NPY and are actually increased in mice lacking the NPY Y1 or Y5 receptors (Erickson, Clegg et al. 1996; Marsh, Hollopeter et al. 1998; Palmiter, Erickson et al. 1998; Pedrazzini, Seydoux et al. 1998). The role of NPY pathways in the fetus is even less clear since the fetus cannot increase energy intake in response to NPY, though other components of the effector systems regulating body adipose stores are likely to be functional. An increase in plasma ACTH and cortisol concentrations in response to NPY leads to peripheral insulin resistance and helps mobilise metabolic substrate at times of energy restriction. Potentially, activation of the fetal HPA axis in late gestation can be interpreted as a metabolic response to failing energy supply, but without NPY antagonist studies it is impossible to assess the contribution of NPY pathways to the preparturient increase in ACTH and cortisol secretion.

7

Discussion

7.1 Summary of findings

The studies reported in this thesis have examined aspects of the central regulation of the adrenal gland during fetal life. Excitatory amino acid neurotransmitters acting through the NMDA receptor are shown to be important for basal ACTH secretion in late gestation. The NMDA receptor probably regulates ACTH secretion through CRH neurons since antagonism of the NMDA receptor attenuated the ACTH response to insulin induced hypoglycaemia without affecting the release of AVP at the median eminence, though it remains possible that endogenous excitatory amino acids act directly at the pituitary. The contribution of peripheral signals to HPA axis activity has also been investigated. Estrogen infusion was without effect on basal ACTH or cortisol concentrations, pituitary sensitivity to AVP and CRH, and the ACTH response to NMDA demonstrating that the late gestation increase in HPA axis activity and response to NMDA is not due to placental estrogen. Peripheral metabolic signals, however, can potentially drive the late gestation increase in HPA axis activity through arcuate nucleus NPY pathways as the HPA axis is able to respond to central NPY from at least day 125 gestation.

7.2 The NMDA receptor and ACTH secretion in the fetus

The marked decline in basal plasma ACTH concentrations with NMDA receptor antagonism in the final few days before parturition confirms a

prominent role for endogenous excitatory amino acid neurotransmitters in the preparturient surge in HPA axis activity. Coupled with the earlier observation that the ACTH response to bolus administration of NMDA increases with gestation (Brooks and Howe 1996), these findings identify the NMDA receptor as an important molecular locus determining the timing of parturition in the sheep fetus.

Exactly what role the NMDA receptor has in the modulation of neuroendocrine secretion is unclear at a cellular level. Glutamate receptors mediate most of the fast excitatory synaptic transmission in the adult central nervous system (Seeburg, Burnashev et al. 1995; Ozawa, Kamiya et al. 1998). Based on pharmacological and molecular analysis, ion-channel coupled excitatory amino acid receptors are subdivided into two broad classes: NMDA and non-NMDA receptors, and both types of receptor are clustered in the post-synaptic membrane at central synapses (Seeburg, Burnashev et al. 1995; Ozawa, Kamiya et al. 1998; Paas 1998).

Uniquely, the ion channel of the NMDA receptor at normal resting membrane potential is blocked by a magnesium ion, and so is prevented from acting as a calcium channel. If, however, the post-synaptic cell is depolarised the magnesium ion is expelled from the ion channel, and the NMDA receptor is free to conduct calcium ions once it has been activated by glutamate. That is, the NMDA receptor can only function as an ion channel if the postsynaptic neuron has already been depolarised (Seeburg, Burnashev et al. 1995; Ozawa, Kamiya et al. 1998). In this way the NMDA receptor functions as a molecular co-incidence detector that recognises when the presynaptic neuron (which releases glutamate) is active at the same time as the post-synaptic neuron has been depolarised by another synaptic input. The entry of calcium into the post-synaptic cell through the NMDA receptor is able, through calcium dependent kinases, to effect longer lasting changes in the function of the synapse and this modulation of synapse function is termed synaptic plasticity.

Secretion of releasing factor at the hypothalamus occurs in short bursts of neurosectory activity, at least for LHRH neurons and for magnocellular AVP and oxytocin neurons (Lincoln, Fraser et al. 1985). It is

difficult to comprehend a role for the NMDA receptor in the acute modulation of neurosecretory activity during the generation say of an LHRH pulse, but certainly changes in synaptic efficiency could underlie the more sustained secretion in particular physiological circumstances such as the LHRH surge at ovulation, or of AVP secretion during dehydration. Interestingly, however, the NMDA receptor may have rather different functions in the fetal brain, and indeed may not be so readily blocked by the magnesium ion. It seems that during development NMDA receptors are expressed before non-NMDA receptors, so at particular points in development the NMDA receptor must mediate much of the excitatory neurotransmission (Wu, Malinow et al. 1996; Golshani, Warren et al. 1998). Additionally, the subunits of the NMDA receptor expressed in the developing brain are such that magnesium provides little block to ion conductance (Ozawa, Kamiya et al. 1998). Such factors could account for the major role of the NMDA receptor in regulating ACTH secretion in the fetus.

Earlier work has shown that the ACTH response to NMDA increases with gestation (Brooks and Howe 1996). There are a number of potential explanations. Firstly, there may be a maturational increase in the number or affinity of NMDA receptors. In other brain regions there is a well characterised developmental change in the subunits of the NMDA receptor that are expressed (Farrant, Feldmeyer et al. 1994; Gottmann, Mehrle et al. 1997). Different subunit compositions of the receptor are known to produce different ion channel kinetics (Ozawa, Kamiya et al. 1998; Paas 1998). An alternative explanation is that the number or peptide content of the CRH and AVP neurons stimulated by NMDA increases. In other words, that there is a greater releasable pool of CRH and AVP in late gestation. The reported changes in total hypothalamic CRH and AVP content, however, do not parallel the gestational increases in NMDA responsiveness, suggesting that this is not the correct explanation. Hypothalamic immunoreactive CRH and AVP content reaches a plateau around day 135 and declines somewhat to term (Brieu, Tonon et al. 1989; Watabe, Levidiotis et al. 1991; Currie and Brooks 1992; Keiger, O'Steen et al. 1994). Increased pituitary sensitivity to AVP and or CRH could account for the increase in ACTH release, but here

too the evidence is that sensitivity declines in late gestation (Norman, Lye et al. 1985; Norman and Challis 1987). Finally, exogenous NMDA acting directly on the pituitary could account for the progressive increase in ACTH release with gestation. Certainly pituitary POMC message concentrations increase with gestation, suggesting there is a greater releasable pool, though message does not always equate with peptide content. Ultimately, the basis for the increasing response to NMDA with gestation cannot be resolved without studies specifically examining the ontogeny of the NMDA receptor in the PVN and associated pathways.

Equally since the drive provided by endogenous excitatory amino acid neurotransmitters has not been probed at different gestations (by measuring the decline in basal ACTH secretion in response to antagonist) it is not possible to comment upon the idea that increasing ACTH secretion reflects increasing release of transmitter. When originally envisaged, the microdialysis studies were intended to look at glutamate release in the PVN and other hypothalamic nuclei. Lack of suitable stereotaxic co-ordinates in our fetal sheep would have necessitated long preliminary studies to define the location of the PVN, and instead it was decided to concentrate upon median eminence AVP and CRH secretion. The low and unchanging concentrations of glutamate at the median eminence indicate that this is not the principal site at which endogenous excitatory amino acids regulate AVP or CRH neurosecretion. Clearly, further studies are required to assess the possibility of increasing glutaminergic transmission in the PVN providing the drive to ACTH secretion in late gestation.

7.3 The NMDA receptor and neuroendocrine development

Following largely genetically programmed early events establishing synaptic connections between neurons, often over considerable distances, functional activity of the neurons becomes important for the maintenance of appropriate connections. It is thought that initially incoming axons establish multiple synapses on nearby neurons and subsequently inappropriate connections are withdrawn while correct synapses persist (Goodman and

Shatz 1993). The persistence of synapses depends upon trophic factors released by the post-synaptic cell in response to neural activity (Patterson and Hiroyuki 1993; Davies 1994; Lindsay, Wiegrand et al. 1994).

Long term changes in synaptic function bought about through the NMDA receptor seem tailor made for the formation and stabilisation of appropriate synaptic connections during development. Activation of the NMDA receptor is thought to stabilise developing synaptic connections and inhibit neuronal apoptosis (Wu, Malinow et al. 1996; Zhang, Rubin et al. 1998). The NMDA receptor is expressed at excitatory synapses before non-NMDA receptors in the central nervous system of the tadpole and rat (Wu, Malinow et al. 1996; Golshani, Warren et al. 1998) whilst in the developing mammalian cerebellum and hippocampus the NMDA receptor subtype expressed is developmentally regulated and correlates to the appearance of a mature pattern of nerve activity (Farrant, Feldmeyer et al. 1994; Gottmann, Mehrle et al. 1997). Blockade of the NMDA receptor disrupts the normal elimination of excess synapses in the cerebellum (Rabacchi, Bailly et al. 1992). Similar events probably occur during development of the neuroendocrine hypothalamus.

Interesting parallels can be drawn between the fetal HPA axis and the hypothalamo-pituitary gonadal axis at puberty. Pubertal activation of the HPG axis is associated with increasing LH responsiveness to NMDA (Cicero, Meyer et al. 1988; Carbone, Szwarcfarb et al. 1992). Pulses of NMDA advance puberty (Urbanski and Ojeda 1987; Plant, Gray et al. 1989) while NMDA receptor blockade delays puberty (Urbanski and Ojeda 1990; Wu, Howe et al. 1990). During pubertal maturation there is increasing release of excitatory amino acid transmitters within the preoptic area as measured by push-pull perfusion and by release from in vitro explants (Goroll, Arias et al. 1993; Carbone, Szwarcfarb et al. 1995). The onset of LHRH pulse generator activity at puberty is thought to be dependent upon maturation of the neuronal systems controlling LHRH release (Carbone, Szwarcfarb et al. 1995; Perera and Plant 1997) and it is thought that exogenous pulses of NMDA may promote the maturation of these pathways (Smyth and Wilkinson 1994). The NMDA receptor may have a comparable influence on the functional

maturation of the HPA axis during critical periods in fetal development. There are, however, no studies on the maturation of synaptic connections onto AVP and CRH neurons in late gestation.

7.4 Metabolic signals and parturition

If the NMDA receptor is involved in the establishment of the correct innervation of the CRH and AVP neurons in the PVN and in longer term regulation of basal hormone secretion, then the role of endogenous excitatory amino acid neurotransmitters in the fetal hypothalamus might be seen as permissive. In other words, the activation of the HPA axis in late gestation is dependent upon the maturation of the neuronal circuitry regulating the PVN and upon the enhancement of synaptic inputs to PVN neurons. What inputs, then, may ultimately determine activation of the HPA axis? A metabolic signal is an attractive candidate for matching the timing of birth to the growth of the fetus and the ability of the placenta to sustain intrauterine growth. Crucial to this hypothesis is evidence for a decline in oxygen, or metabolic substrate in later gestation. In fact, there is a surprising paucity of information about longitudinal changes in metabolite concentrations in fetal plasma during pregnancy. In cross sectional studies of small and appropriately grown human fetuses, no change in glucose concentration was demonstrated with gestation, though the fetal glucose/insulin ratio increased exponentially (Economides and Nicolaides 1989; Economides, Proudler et al. 1989). At the same time there was a decline in the concentration of amino acids in fetal plasma implying increasing consumption by the fetus or placenta (Economides, Nicolaides et al. 1989). There is also a gestation related fall in the partial pressure of oxygen and an increase in the partial pressure of carbon dioxide in the fetal circulation (Nicolaides, Economides et al. 1989). In other words, the in utero environment probably does become more hostile near the end of pregnancy and could provide the drive leading to parturition. This hypothesis can only really be tested by restoring the late gestation in utero environment to a state found earlier in gestation. Circumstantial evidence can be garnered by examining the changes in the central pathways

likely to be involved in responding to metabolic and hypoxic stress, and attempting to manipulate these pathways.

The arcuate nucleus NPY system is likely to be part of the central pathway mediating metabolic feedback. Others have shown an increase in the expression of message for NPY in the arcuate nucleus in the sheep fetus with gestation, and further, that NPY message expression is increased by maternal undernutrition (Warnes, Morris et al. 1998). The demonstration that central administration of NPY stimulates ACTH secretion in late gestation confirms the potential for this pathway to drive the HPA axis. Studies with NPY antisera or antagonists, however, are needed to establish the contribution of endogenous NPY pathways to parturition.

7.5 Steroid regulation of the NMDA receptor

The factors regulating the pattern of NMDA receptor subunit expression during development are unknown. Evidence from the adult hypothalamus suggests that gonadal steroids modulate both release of transmitter (Carbone, Szwarcfarb et al. 1992; Carbone, Szwarcfarb et al. 1995) and expression of the NMDA receptor (Weiland 1992; Gazzaley, Weiland et al. 1996; Woolley, Weiland et al. 1997), though not all studies have found the NMDA receptor to be estradiol sensitive. A lack of estradiol sensitivity is reported in studies of glutamate receptors in adult hippocampus and temporal cortex (Kohama, Garyfallou et al. 1998) and hypothalamus (Brann, Zamorano et al. 1993). No previous studies have specifically examined the effects of estrogen on the sensitivity of the fetal HPA axis to NMDA, though it has been demonstrated that in primates (Baggia, Albrecht et al. 1990; Pepe, Waddell et al. 1990; Pepe, Davies et al. 1994) and sheep (Saoud and Wood 1997; Wood and Saoud 1997) that estrogen will increase basal HPA axis activity.

The studies described in chapter 5 indicate that estradiol at biologically active concentrations does not increase HPA axis sensitivity to NMDA in the sheep fetus. In addition, the estradiol infusion failed to elevate basal HPA axis activity in contradiction to the previous results. The failure of estradiol to

activate the fetal HPA axis is reported in one other study (Wang, Matthews et al. 1997). The reasons for the differences between the fetal sheep studies are unclear but relate to route of administration, since dosages and gestation are otherwise similar. The two studies from Woods laboratory utilise an estradiol implant that is presumed to release at a constant rate over several days, wheras the work in chapter 5, and that of Wang are based on measured infusion of estradiol. The results reported in chapter 5 and those of Wang suggest that at physiological levels found prior to the onset of labour it is unlikely that estradiol significantly increases either basal ACTH secretion or the response to NMDA.

Another candidate for increasing expression of the NMDA receptor is cortisol (Weiland, Orchinik et al. 1997). And in this regard, recent experiments have demonstrated the potential for sustained cortisol infusion to lead to increased ACTH secretion (Apostolakis, Longo et al. 1994) but further studies are required to examine the role of the NMDA receptor in this phenomenon. Steroids almost certainly modulate NMDA receptor subunit expression indirectly through regulation of other transcription factors capable of binding the upstream promoter of the various NMDA receptor subunits (Bai and Kusiak 1997; Bai, Norton et al. 1998; Klein, Pieri et al. 1998; Kranic, Bai et al. 1998) but this is a complex and poorly understood area beyond the remit of this discussion.

7.6 Conclusions

The NMDA receptor offers a defined locus important for determining basal ACTH secretion in late gestation. The NMDA receptor is probably coupled to ACTH secretion through CRH neurons, but this conclusion requires more direct confirmation. The factors regulating the expression of the NMDA receptor and the development of the neural pathways controlling HPA axis activity in the fetus remain unknown. In other situations the NMDA receptor is implicated in the development of nerve pathways, and it is tempting to speculate that the increased sensitivity of the fetal HPA axis to NMDA in late gestation reflects maturation of the nerve pathways regulating

ACTH secretion. The timing of parturition is then dependent both upon maturation of the nerve pathways and increasing activity in these pathways. Potentially metabolic cues may drive increased ACTH secretion, and in this regard central NPY containing pathways are capable of stimulating ACTH secretion in late gestation. Further experiments are needed, however, to substantiate the hypothesis that parturition is triggered by failing metabolite supply and reduced fetal growth.

Appendix 1

Reagents for radioimmunoassay

Reagent	Supplier
Acetic acid,	Sigma
Acetone,	Sigma
Bovine serum albumin fraction V,	Sigma
Chloramine-T,	BDH
Diaminobenzidine ,	Sigma
EDTA,	Sigma
Hydrogen peroxide,	Sigma
Methanol,	Sigma
Polypep,	Sigma
Polyethylene glycol-6000,	BDH
Sephadex G100	Pharmacia
Sodium azide,	BDH
Sodium chloride,	BDH
Sodium dihydrogen orthophosphate dihydrate,	BDH
Sodium hydrogen carbonate,	BDH
di-Sodium hydrogen phosphate anhydrous,	BDH
Sodium iodide (^{125}I)	Amersham
Sodium Metabisulphite ,	BDH
Thiomersal,	BDH
Trifluoroacetic acid,	Sigma
Triton X-100.	BDH

Appendix 2

Publications arising

Publications

Brooks AN, **Howe DC**, Porter DWF, Naylor AM (1994) Neuropeptide-Y stimulates pituitary-adrenal activity in fetal and adult sheep. Journal Neuroendocrinology 6:161-166

Brooks AN, **Howe DC** (1996) Adrenocorticotrophin and luteinising hormone responses to N-Methyl-D-Aspartate during fetal development in sheep. Journal Neuroendocrinology 8:315-321

A.N. Brooks AN, Hagan DM, **Howe DC** (1996) Neuroendocrine regulation of pituitary-adrenal function during fetal life. European Journal Endocrinology 135:153-165

Abstracts and oral communications (Published proceedings)

Antagonism of the N-methyl-D-Aspartate (NMDA) receptor inhibits adrenocorticotrophin (ACTH) secretion in the late gestation ovine fetus. D.C.Howe & A.N.Brooks. Oral communication. British Neuroendocrine Group, Manchester, 1995

Microdialysis as a tool for investigating the hypothalamic control of adrenocorticotropin (ACTH) secretion in the ovine fetus. D.C.Howe & A.N.Brooks. Poster. American Endocrine Society, Washington, June 1995.

Differential coupling of N-Methyl-D-Aspartate (NMDA) receptor to adrenocorticotrophin (ACTH) and growth hormone (GH) secretion in the late gestation ovine fetus. D.C.Howe & A.N.Brooks. Poster. Society for the Study of Fertility, Dublin, July 1995.

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